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(54) Title: NOVEL ADMINISTRATION OF THROMBOPOIETIN

(57) Abstract

Thrombopoietin materials can be administered with substantial therapeutic effect in a single or low–multiple daily administration. Reversal of thrombocytopenia is achieved by administering to a patient having or in need of such treatment a single or low–multiple daily dose of a therapeutically effective amount of a thrombopoietin.

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NOVEL ADMINISTRATION OF THROMBOPOIETIN

Field of the Invention:

The present invention relates to a new method of using thrombopoietin, and biologically active derivatives and isoforms thereof, for the treatment of immune and/or hematopoietic disorders including thrombocytopenia. The use contemplates the co-administration of such materials together with a cytokine, especially a colony stimulating factor or interleukin. The use includes and is included within a method for treating a mammal having or at risk for thrombocytopenia by administering to the mammal in need of such treatment a therapeutically effective amount of the material(s).

Background of the Invention:

The hematopoietic system produces the mature highly specialized blood cells known to be necessary for survival of all mammals. These mature cells include erythrocytes, specialized to transport oxygen and carbon dioxide, T- and B-lymphocytes, responsible for cell- and antibody-mediated immune responses, platelets or thrombocytes, specialized to form blood clots, and granulocytes and macrophages, specialized as scavengers and as accessory cells to combat infection. All of these specialized mature blood cells are derived from a single common primitive cell type referred to as the pluripotent stem cell found primarily in bone marrow.

The mature highly specialized blood cells must be produced in large numbers continuously throughout the life of a mammal. The vast majority of these specialized blood cells are destined to remain functionally active for only a few hours to weeks. Thus, continuous renewal of these mature blood cells, the primitive stem cells themselves, as well as any intermediate or lineage, committed progenitor cell lines lined between the primitive and mature cells, is necessary in order to maintain the normal steady state blood cell needs for continued life of the mammal.

At the heart of the hematopoietic system lies the pluripotent stem cell(s). These cells are relatively few in number and undergo self-renewal by proliferation to produce daughter stem cells, or they are transformed in a series of differentiation steps into increasingly mature lineage-restricted progenitor cells, ultimately forming the highly specialized mature blood cell(s).

The underlying principal of the normal hematopoietic cell system appears to be decreased capacity for self-renewal as multipotency is lost and lineage-restriction and maturity is acquired. Thus, at one end of the hematopoietic cell spectrum lies the pluripotent stem cell possessing the capacity for self-renewal and differentiation into all the various lineage-specific committed progenitor cells. At the other end of the spectrum lie the highly lineage-restricted progenitors and their progeny which have lost the ability of self renewal but have acquired mature functional activity.

The proliferation and development of stem cells and lineage-restricted progenitor cells are carefully controlled by a variety of hematopoietic growth factors or cytokines. Thus, hematopoietic growth factors may influence growth and differentiation of one or more lineages, may overlap with

other growth factors in affecting a single progenitor cell-line, or may act synergistically with other factors.

It will be appreciated from the foregoing that novel hematopoietic growth factors that effect survival, proliferation, differentiation or maturation of any of the blood cells or predecessors thereof would be useful, especially to assist in the re-establishment of a diminished hematopoietic system caused by disease or after radiation- or chemo-therapy.

Platelets are critical elements of the blood clotting mechanism. Depletion of the circulating level of platelets, called thrombocytopenia, occurs and is manifested in various clinical conditions and disorders. Clinical thrombocytopenia is commonly defined as a condition wherein the platelet count is below about 150×10^9 per liter. The major causes of thrombocytopenia can be broadly divided into three categories on the basis of platelet life span, namely: 1) impaired production of platelets by the bone marrow, e.g., thrombocytopenia brought about by chemo- and radiation-therapy, 2) platelet sequestration in the spleen (splenomegaly) and 3) increased destruction of platelets in the peripheral circulation, e.g., thrombocytopenia brought about by autoimmune disorders. Additionally, in patients receiving large volumes of rapidly administered platelet-poor blood products, thrombocytopenia may develop due to dilution factors. A more detailed description of thrombocytopenia and its causes, may be found in Schafner, "Thrombocytopenia and Disorders of Platelet Disfunction", Internal Medicine, John J. Hutton *et al.* Eds., Little, Brown & Co., Boston/Toronto/London, Third Ed. (1990) as well as International Patent Application No. PCT/US94/14553 (International Publication No. WO95/18858).

The therapeutic approach to the treatment of patients with thrombocytopenia is dictated by the severity and urgency of the clinical situation. The treatment is similar for HIV-associated and non-HIV-related thrombocytopenia, and although a number of different therapeutic approaches have been used, the therapy remains clinically controversial.

It will be appreciated from the foregoing that one way to treat thrombocytopenia would be to obtain an agent capable of accelerating the differentiation and maturation of megakaryocytes or precursors thereof into the platelet-producing form. Considerable efforts have been expended on identifying such an agent. One commonly referred to is thrombopoietin (TPO), the subject of the present application. Other names for TPO commonly found in the literature at this time include: thrombocytopoiesis stimulating factor (TSF); megakaryocyte colony-stimulating factor (MK-CSF), megakaryocyte growth and development factor (MGDF), megakaryocyte stimulating factor, megakaryocyte potentiator and *mpl* ligand.

The cited International Patent Application PCT/US94/14553 describes the identification, isolation, production and use of an isolated mammalian megakaryocytopoietic proliferation and maturation promoting protein denominated the "MPL ligand" (ML), or more commonly, "thrombopoietin" (TPO), which has been found capable of stimulating proliferation, maturation and/or differentiation of megakaryocytes into the mature platelet-producing form.

Attention is directed as well to International Patent Application Publications Nos. WO95/26746, WO95/21919 and WO95/21920.

The PCT/US94/14553 application includes various aspects of associated embodiments of TPO, including a method of treating a mammal having or at risk for a hematopoietic disorder, notably thrombocytopenia, comprising administering a therapeutically effective amount of TPO materials to the mammal. Optionally, TPO is administered as such or in combination with a cytokine, especially a colony stimulating factor or interleukin. For purposes disclosed in the International Patent Application, TPO is broadly defined as including TPO itself or various variants, derivatives or isoforms thereof, including fragments that share at least one biological property in common with intact TPO for the treatment of thrombocytopenia. "Biological property", when used in conjunction with the definition of the various TPO materials useful as described in the patent application, means that they have thrombopoietic activity or an in vivo effector or antigenic function or activity that is directly or indirectly caused or performed by the TPO material.

With respect to the therapeutic use of thrombopoietin materials, as described in the International Patent Application No. PCT/US94/14553, the TPO materials are therein described for administration in admixture with a pharmaceutically acceptable carrier via any of several administrative modes. The daily regimen is described as ranging from about 0.1 to 100 microgram/kilogram body weight, preferably from about 0.1 to 50 microgram/kg body weight, preferably at an initial dosage ranging from about 1 to 5 microgram/kg per day. Implicit within the teachings of the patent application is a regimen of administering such a dosage rate over a period of several to many days following a projected or actual state of reduced platelet count.

Published clinical studies of clinically administered thrombopoietin indicates a dosage and administration regimen consisting of the administration of thrombopoietin, subcutaneously at dosages of 0.03 to 5.0 microgram/kg body weight once per day over a period of ten days for a condition marked by thrombocytopenia. See Abstract 1977, *Blood 86* (1995). See also Abstracts 1012, 1014 and 1978, *Blood 86* (1995).

A single injection of pegylated (PEG) murine megakarocyte growth and development factor (mMGDF) into mice is sufficient to produce a stimulation of megakaryocyte frequency, size and ploidization. The PEG-mMGDF was administered to mice at a dose of 25 micrograms/kg as a single intravenous injection. Blood, Feb. 1, 1997, 89(3):823-33). The in vivo effects of PEG-rhMGDF on hematopoiesis in normal mice is reported in Stem Cells, Nov. 1996, 14(6):651-60. See also Blood, Jul. 15, 1996, 88(2):511-21 and Blood, Jun. 15, 1996, 87(12):5006-15.

The effects of rhTPO on myelosuppressive chemotherapy-induced thrombocytopenia in monkeys is reported in Brit. J. Haematol., Sept. 1996, 94(4):722-8. After treatment with nimustine on day 0, monkeys intravenously received rhTPO at a dose of 0.04, 0.2 or 1.0 microgram/kg/d. Administration of rhTPO following nimustine treatment reduced the severity of thrombocytopenia and accelerated the rate of platelet recovery in a dose dependent fashion. The thrombopoietic effects of

PEG-rhMGDF in human patients with advanced cancer are reported in Lancet, Nov. 9, 1996, 348(9037):1279-81. In this study, PEG-rhMGDF was given by subcutaneous injection at a dose of 0.03, 0.1, 0.3 or 1.0 microgram/kg body weight before chemotherapy. Further, the effects of PEG-rhMGDF on platelet counts after chemotherapy for lung cancer are reported in New Engl. J. Med., Feb. 6, 1997, 336(6):404-9 and the effects of this compound injected subcutaneously into rhesus macaques receiving intense marrow suppression by hepsulfam treatment is reported in Blood, Jan. 1, 1997, 89(1):15565.

Likewise, the compound epoetin alfa, which is a given name for erythropoietin (marketed as EPOGEN by Amgen, Inc.), is a glycoprotein indicated for stimulation of red blood cell production. It is indicated in a dosage and administration regimen consisting of starting doses over a range of 150 to 300 units per kg three times weekly for a period of many weeks in order to stimulate the proliferation of red blood cells in patients suffering from a depletion however realized.

G-CSF and GM-CSF are cytokines which induce cycling and increase proliferation of myeloid progenitor cells. The pharmacokinetics of these cytokines have been studied and, different administration regimens have been proposed for each of these drugs when used in conjunction with chemotherapy.

Filgastrium, marketed as NEUPOGEN by Amgen, Inc., is a granulocyte colony stimulating factor (G-CSF). Its indicated regimen is the administration of from 5 to 10 microgram/kg subcutaneously daily for two weeks following chemotherapy. G-CSF is contra-indicated for prechemotherapy administration. Clinical trials in which G-CSF was administered after chemotherapy and also both before and after chemotherapy have shown that preadministration worsened the toxic effects of the chemotherapeutic agent on the bone marrow. J. Nat. Canc. Inst.,1996, vol. 88, No. 19 and Exp. Hemat., 1994, 22:100-102.

GM-CSF has also been studied clinically for use in conjunction with chemotherapy. In contrast to G-CSF, GM-CSF has a relatively short effective half-life. Administration of GM-CSF is followed by a rapid increase in the proliferative activity of the hematopoietic precursors. However, within 72 hours after suspension of administration, a negative feedback is established resulting in a reduction of the proliferative activity of the marrow to values below baseline. The short half-life of GM-CSF has enabled this cytokine to be administered prior to chemotherapy. Cancer, 1993, vol. 72, No. 10.

The conventional regimen in administering materials for the proliferation of red blood cells or other primary blood cells to reverse the effects of thrombocytopenia, is continuous administration of therapeutically effective amounts of the biological material daily over a period of many days to patients in need of such therapy following chemotherapy resulting in thrombocytopenia. While GM-CSF may have limited effectiveness when administered prior to chemotherapy, G-CSF worsens patient thrombocytopenia when administered prior to chemotherapy. The administration of cytokines having a relatively long half-life and which start hematopoietic progenitor cells cycling and proliferating, prior

to treatment with radiation or a chemotherapeutic agent has generally been contra-indicated since these cytoreductive treatments kill not only malignant cells, but also the proliferating progenitor cells as well.

Another approach to the treatment of patients with thrombocytopenia or who are at risk of thrombocytopenia as a result of a medical procedure, e.g. radiation and/or chemotherapy, is to rescue the patient with an autologous hematopoietic implant. In this approach, patients are administered a compound which mobilizes peripheral blood hematopoietic progenitor cells prior to the medical treatment which will induce thrombocytopenia. The mobilized progenitor cells are harvested by known leukapheresis procedures and then retransplanted into the patient after the onset of thrombocytopenia in order to reestablish the patients autologous hematopoietic cells in the bone marrow. Unfortunately, many patients undergoing mobilization have very low numbers of progenitor cells at the time of harvest, necessitating multiple leukopheresis procedures which is painful and inconvenient for the patient. A method which improves the mobilization of progenitor cells thereby reducing the number of leukopheresis procedures is therefore highly desirable.

For convenience to physicians and patients alike, there exists an objective of developing alternative dosage/administration regimens of cytokines materials that would be advantageous and therapeutically equivalent or superior to reverse the effects of thrombocytopenia.

Summary of the Invention

One object of the present invention, therefore, is to provide a method of administering a thrombopoietin which provides improved recovery from thrombocytopenia and overcomes the deficiencies noted above for existing methods of administering cytokines.

Another object is to provide a method of administering a thrombopoietin to a mammal or patient receiving radiation and/or chemotherapy treatment which minimizes thrombocytopenia associated with such treatment and reduces the need for platelet transfusions in the mammal.

These and other objects which will become apparent during the course of the following descriptions of exemplary embodiments have been achieved by the method of the present invention.

The present invention is based upon the unexpected and surprising finding that biologically active thrombopoietin materials can produce therapeutic effect by administering a single or low-multiple daily dose of a therapeutically effective amount to a patient having or in need of treatment for thrombocytopenia. This finding is based on a finding that thrombopoietin materials are growth factors for and are believed to act directly on early bone marrow stem cells and megakaryocyte progenitor cells, in contrast to G-CSF and GM-CSF which are thought to act on progenitor cells later in the hematopoietic cell lineage. The materials of the invention are capable of causing megakaryocyte differentiation of stem cells and increasing platelet count following administration. They induce proliferation and differentiation of bone marrow hematopoietic cells, increasing the number of mature megakaryocytes, which yield increased numbers of circulating platlets.

Thus, the present invention is directed to a method of treating a mammal having or at risk for thrombocytopenia comprising administering to a mammal in need of such treatment a single or low-multiple daily dose of a therapeutically effective amount of a thrombopoietin. In one aspect, the present invention is directed to the single administration of a therapeutically effective amount of a thrombopoietin to such a mammal.

In another aspect of this embodiment, the invention concerns the administration of a thrombopoietin to a mammal which receives at least one cycle of radiation and/or chemotherapeutic agent in need of such a cycle. Typically, the mammal will need one or more of such a cycle for the treatment of a tumor, malignancy, etc. In another aspect, the invention is directed to a method of reducing the number of platelet transfusions in a thrombocytopenic patient. In a further aspect, the invention is directed to a method of mobilizing progenitor cells by the administration of a single or low-multiple daily dose of an effective amount of a thrombopoietin.

Brief Description of the Drawings

Figure 1 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected subcutaneously with 0.1 microgram rmTPO(335) for 1,2, 4, or 8 days. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 2 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected subcutaneously with a single dose at various levels of rmTPO(335) 24 hours after the initiation of the experiment. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 3 - Log-linear representations of the platelet (panel A) and erythrocyte (panel B) responses to single administrations of rmTPO(335) given either subcutaneously or intravenously in animals rendered pancytopenic by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg). The cell numbers plotted are those measured on day 14 after initiation of the experiment. $\overline{\underline{0}}$ is base line zero level.

Figure 4 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected intravenously with a single dose at various levels of rmTPO(335) 24 hours after the initiation of the experiment. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 5 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected subcutaneously with a single dose at 24 hours after the initiation of the experiment with various forms of rmTPO(153) conjugated to polyethylene

glycol (peg) of either 20K or 40K molecular weight. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 6 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected subcutaneously with a single dose at 24 hours after the initiation of the experiment with either rmTPO(335) or rmTPO(153) conjugated to polyethylene glycol (peg) of 40K molecular weight. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 7 - Animals rendered pancytopenic, by a combination of 5.0 Gy of γ-irradiation and carboplatin (1.2 mg), were injected intravenously with a single dose at 24 hours after the initiation of the experiment with either rmTPO(335) or rmTPO(153) conjugated to polyethylene glycol (peg) of 40K molecular weight. Panel A shows the platelet response to the treatment regimens while panels B and C represent the erythrocyte and leukocyte responses respectively over a 28 day period. The key set forth in panel B refers to all three panels.

Figure 8 - Figure 8 shows the thrombocyte level of 6 Gy irradiated mice at the time of nadir in placebo controls as a function of the time of administration of a single i.p. dose (0.3 microgram) of TPO at each of the various time points indicated in the Figure.

Figure 9 - Figure 9 shows the thrombocyte level of 6 Gy irradiated mice at the time of nadir in placebo controls as a function of the time of administration of a single i.p. dose (30 microgram) of TPO at two hours before (-2h) irradiation.

Figure 10 - In order to model a protracted form of cytoreductive treatment very similar to radiation or chemotherapy, total body irradiation (TBI) was given to mice in three equal fractions of 3 Gy separated by 24 hours each. TPO was given in a total dose of 0.9 microgram in three different dosing regimen; 3 x 0.3 microgram at +2h from irradiation, 0.9 microgram at +2h from irradiation, and 0.9 microgram at -2h from irradiation. The resulting thrombocyte levels are shown vs a placebo.

Figure 11 - This Figure shows hemopoietic progenitor cell data of the femur for the regimen of Figure 10.

Figure 12 - This Figure shows hemopoietic progenitor cell data of the spleen for the regimen of Figure 10.

Figure 13 - Figure 13 shows pharmacokinetic data following three doses of 0.3 microgram or a single dose of 0.9 microgram of TPO.

Figure 14 - Figure 14 shows median platelet counts averaged over dose levels by study arm for Example 7, cycle 1 (chemotherapy alone).

Figure 15 - Figure 15 shows median platelet counts averaged over dose levels by study arm for Example 7, cycle 2 (chemotherapy and rhTPO).

Figure 16 - Figure 16 shows median platelet counts averaged over dose levels by study arm for Example 7, cycle 3 (chemotherapy and rhTPO).

Figure 17 - Figure 17 shows median platelet counts averaged over dose levels by study arm for Example 7, cycle 4 (chemotherapy and rhTPO).

Figure 18 - Figure 18 shows median platelet count by rhTPO dose level for arm C, cycle 2 of Example 7.

Figure 19 - Figure 19 shows median platelet count by rhTPO dose level for arm D, cycle 2 of Example 7.

Figure 20 - Platelet counts in mice exposed to a single inhalation of rhTPO; see Example 8.

Figure 21 - Platelet counts in mice exposed to multiple inhalations of rhTPO; see Example 8.

Figure 22 - Expansion of $CD34^+$ cells by TPO/FL/KL. In Figure 22 is shown the expansion of $CD34^+$ cells over 8 weeks in cultures containing TPO, Flt-3 and c-kit ligand. An expansion of over a 10e6 fold is observed; see Example 14.

Figure 23 - Expansion of $CD34^+CD38^-$ cells by TPO/KL/FL. As shown in Figure 23 the subpopulation of $CD34^+CD38^-$ cells is also expanded. At week one this subpopulation only makes up only 8% of the culture but by week 8 comprises 33% of the culture, indicating a 4 fold expansion. This demonstrates both an expansion and maintanence of a primitive progenitor population in these expanded cultures; see Example 14.

Figure 24 - Expansion of multilineage activity by TPO/KL/FL. In Figure 24 the ability of the expanded cultures to give rise to multilineage colonies in vitro is shown. The number of colonies generated increases porportionally with the expansion of $CD34^+$ cells in the culture. This indicates that the expanded cells are maintaining their multipotential activity; see Example 14.

Definitions

As used herein, a "mammal having or at risk for thrombocytopenia" means a mammal, including a human, which is experiencing thrombocytopenia, that is, a platelet count which is below the platelet count for average normal individuals in the mammal population. In humans, thrombocytopenia is defined as a condition where the platelet count is below about 150×10^9 per liter of blood. The mammal may, however, also be at risk for thrombocytopenia, meaning that the mammal may forseeably experience a thrombocytopenic condition as a result of a specific treatment which is known to cause thrombocytopenia. For example, a mammal is at risk for thrombocytopenia if the mammal will be administered a radiation and/or chemotherapeutic treatment which is known to induce thrombocytopenia in the treated mammal. In other words, it is clearly forseeable that the mammal is at risk for or has a high probability of experiencing thrombocytopenia as a result of the treatment which is known to induce thrombocytopenia. Such mammals at risk for thrombocytopenia may be treated with the method of the present invention. Included within the scope of this invention are mammals having or

at risk for thrombocytopenia as a result of a disfunctional liver, e.g. liver cirrhosis, and mammals undergoing progenitor cell mobilization therapy and apheresis, generally prior to radiation and/or chemotherapy treatment.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone, insulin-like growth factors, human growth hormone including N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and leutinizing hormone (LH), hematopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factors (TNF-α and TNF-β), mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, nerve growth factors (NGFs) such as NGF-β, insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons (IFNs) such as interferon-α, -β and -γ, colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF), interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 and other polypeptide factors including LIF, SCF, FLT-3 ligand and kit-ligand (KL). As used herein the foregoing terms are meant to include proteins from natural sources or from recombinant cell culture. Similarly, the terms are intended to include biologically active equivalents; e.g., differing in amino acid sequence by one or more amino acids or in type or extent of glycosylation.

The term "biologically active" when used in conjunction with a thrombopoietin (TPO) means thrombopoietin or a thrombopoietic polypeptide that exhibits thrombopoietic activity or shares an effector function of the *mpl* ligand isolated from aplastic porcine plasma or expressed in recombinant cell culture. A principal known effector function of the *mpl* and stimulating the incorporation of labeled nucleotides (^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P. Another known effector function of the *mpl* ligand or polypeptide herein is the ability to stimulate the incorporation of ^{35}S into circulating platelets in a mouse platelet rebound assay. Yet another known effector function of *mpl* ligand is the ability to stimulate in vitro human megakaryocytopoiesis that may be quantitated by using a radio labeled monoclonal antibody specific to the megakaryocyte glycoprotein $GPII_bIII_a$.

The terms "*mpl* ligand", *mpl* ligand polypeptide", "ML", "thrombopoietin" or "TPO" are used interchangeably herein and include any polypeptide that possesses the property of binding to *mpl*, a member of the cytokine receptor superfamily, and having a biological property of *mpl* ligand. An exemplary biological property is the ability to stimulate the incorporation of labeled nucleotides (e.g. ^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl*. Another

exemplary biological property is the ability to stimulate the incorporation of ^{35}S into circulating platelets in a mouse platelet rebound assay. This definition encompasses a polypeptide isolated from a *mpl* ligand source such as aplastic porcine plasma described herein or from another source, such as another animal species, including humans, or prepared by recombinant or synthetic methods. Examples include TPO(332) and $rhTPO_{332}$. Also included in this definition is the thrombopoietic ligand described in WO 95/28907 having a molecular weight of about 31,000 daltons (31kd) as determined by SDS gel under reducing conditions and 28,000 daltons (28kd) under non-reducing conditions. The term "TPO" includes variant forms, such as fragments, alleles, isoforms, analogues, chimera thereof and mixtures of these forms. For convenience, all of these ligands will be referred to below simply as "TPO" recognizing that all individual ligands and ligand mixtures are referred to by this term.

Preferably, the TPO is a compound having thrombopoietic activity or being capable of increasing serum platelet counts in a mammal. The TPO is preferably capable of increasing endogenous platelet counts by at least 10%, more preferably by 50%, and most preferably capable of elevating platelet counts in a human to greater than about 150×10^9 per liter of blood. The TPO of this invention preferably has at least 70% overall sequence identity with the amino acid sequence of the highly purified substantially homogeneous porcine *mpl* ligand polypeptide and at least 80% sequence identity with the "EPO-domain" of the porcine *mpl* ligand polypeptide. Alternatively, the TPO of this invention may be a mature human *mpl* ligand (hML), or a variant or post-transcriptionally modified form thereof or a protein having about 80% sequence identity with mature human *mpl* ligand. Alternatively, the TPO may be a fragment, especially an amino-terminus or "EPO-domain" fragment, of the mature human *mpl* ligand. Preferably, the amino terminus fragment retains substantially all of the human ML sequence between the first and fourth cysteine residues but may contain substantial additions, deletions or substitutions outside that region. According to this embodiment, the fragment polypeptide may be represented by the formula:

X-hTPO(7-151)-Y

Where hTPO(7-151) represents the human TPO (hML) amino acid sequence from Cys^7 through Cys^{151} inclusive; X represents the amino group of Cys^7 or one or more of the amino-terminus amino acid residue(s) of the mature TPO or amino acid residue extensions thereto such as Met, Lys, Tyr or amino acid substitutions thereof such as arginine to lysine or leader sequences containing, for example, proteolytic cleavage sites (*e.g.* Factor Xa or thrombin); and Y represents the carboxy terminal group of Cys^{151} or one or more carboxy-terminus amino acid residue(s) of the mature TPO or extensions thereto.

A "TPO fragment" means a portion of a naturally occurring mature full length *mpl* ligand or TPO sequence having one or more amino acid residues or carbohydrate units deleted. The deleted amino acid residue(s) may occur anywhere in the peptide including at either the N-terminal or C-

terminal end or internally, so long as the fragment shares at least one biological property in common with *mpl* ligand. *Mpl* ligand fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30 or 40 amino acid residues that are identical to the sequences of the *mpl* ligand isolated from a mammal including the ligand isolated from aplastic porcine plasma or the human or murine ligand, especially the EPO-domain thereof. Representative examples of N-terminal fragments are TPO(153), hML_{153} or TPO(Met^{-1} 1-153).

The terms "TPO isoform(s)" and "TPO sequence isoform(s)" or the term "derivatives" in association with TPO, etc. as used herein means a biologically active material as defined below having less than 100% sequence identity with the TPO isolated from recombinant cell culture, aplastic porcine plasma or the human *mpl* ligand. Ordinarily, a biologically active *mpl* ligand or TPO isoform will have an amino acid sequence having at least about 70% amino acid sequence identity with the *mpl* ligand/TPO isolated from aplastic porcine plasma or the mature murine, human *mpl* ligand or fragments thereof, preferably at least about 75%, more preferably at least about 80%, still more preferably at least about 85%, even more preferably at least about 90%, and most preferably at least about 95%.

In one isoform embodiment, the TPO may have the formula:

SerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSer
HisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProXaaPro
ValLeuLeuProAlaValAspXaaXaaLeuGlyGluTrpLysThrGlnMetGluGlu
ThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAla
AlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGly
GlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGlnXaaXaaXaa
XaaGlyArgThrThrAlaHisXaaAspProAsnAlaIlePheLeuSerPheGlnHis
LeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysVal
ArgArgAlaProProThrThrAlaValProSerArgThrSerLeuValLeuThrLeu
AsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThrAlaSerAla
ArgThrThrGlySerGlyLeuLeuLysXaaGlnGlnGlyPheArgAlaLysIlePro
GlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsnArg
IleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr
LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsn
LeuGlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeu
PheProLeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuPro
AspProSerAlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThr
HisSerGlnAsnLeuSerGlnGluGly

where:

Xaa at position 37 is Thr, Asp or Glu;
Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp, or Met;
Xaa at position 47 is Ser, Asp or Glu;
Xaa at position 112 is deleted or Leu, Ala, Val, Ile, Pro, Phe, Trp, or Met;
Xaa at position 113 is deleted or Pro, Phe, Ala, Val, Leu, Ile, Trp, or Met;
Xaa at position 114 is deleted or Pro, Phe, Ala, Val, Leu, Ile, Trp, or Met;
Xaa at position 115 is deleted or Gln, Gly, Ser, Thr, Tyr, or Asn;
Xaa at position 122 is Lys, Arg, His, Glu, or Asp;
Xaa at position 200 is Trp, Ala, Val, Leu, Ile, Pro, Phe, Met, Arg and Lys, or His.

where from 1 to 179 amino acids can be deleted from the C-terminus and with the proviso that at least one of the amino acids designated by Xaa are different from the corresponding amino acids of the native TPO (1-332).

This embodiment also includes a TPO fragment with the following formula:

SerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSer
HisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProXaaPro
ValLeuLeuProAlaValAspXaaXaaLeuGlyGluTrpLysThrGlnMetGluGlu
ThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAla
AlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGly
GlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGlnXaaXaaXaa
XaaGlyArgThrThrAlaHisXaaAspProAsnAlaIlePheLeuSerPheGlnHis
LeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysVal
Arg

where:

Xaa at position 37 is Thr, Asp or Glu;
Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp, or Met;
Xaa at position 47 is Ser, Asp or Glu;
Xaa at position 112 is deleted or Leu, Ala, Val, Ile, Pro, Phe, Trp, or Met;
Xaa at position 113 is deleted or Pro, Phe, Ala, Val, Leu, Ile, Trp, or Met;
Xaa at position 114 is deleted or Pro, Phe, Ala, Val, Leu, Ile, Trp, or Met;
Xaa at position 115 is deleted or Gln, Gly, Ser, Thr, Tyr, or Asn;
Xaa at position 122 is Lys, Arg, His, Glu, or Asp;

and with the proviso that at least one of the amino acids designated by Xaa is different from the corresponding amino acids of the native TPO (1-332). These variants may have an improved biological profile, such as increased proliferative activity and/or decreased side-effects, and/or improved physical properties, such as improved half-life, stability, and/or re-fold efficiencies. The preparation of the polypeptides of this embodiment is described in WO96/23888.

TPO "analogues" include covalent modification of TPO or *mpl* ligand by linking the TPO polypeptide to one of a variety of nonproteinaceous polymers, *e.g.* polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. TPO polypeptides covalently linked to the forgoing polymers are referred to herein as pegylated TPO.

A "chimeric polypeptide" or "chimera" as used herein is a polypeptide containing full length parent ligand (TPO or *mpl* ligand) or one or more fragments thereof fused or bonded to a second heterologous polypeptide or one or more fragments thereof. The chimera will share at least one biological property in common with TPO. The second polypeptide will typically be a cytokine, for example the cytokines noted above, immunoglobin or fragment thereof. The two polypeptides may be directly bonded together or may be bonded together through a linker, for example a peptide linker which may have 2-50, generally 2-20 amino acid units. Specific examples include TPO/G-CSF, TPO/GM-CSF, TPO/IL-3, TPO/IL-6, etc. Preparation of chimeric proteins may be accomplished using methods well-known in the art.

The term "biological property" when used in conjunction with either the "*mpl* ligand" or "isolated *mpl* ligand" or "TPO" means having thrombopoietic activity or having an in vivo effector or antigenic function or activity that is directly or indirectly caused or performed by a *mpl* ligand or "TPO" (whether in its native or denatured conformation) or a fragment thereof. Effector functions include *mpl* binding and any carrier binding activity, agonism or antagonism of *mpl*, especially transduction of a proliferative signal including replication, DNA regulatory function, modulation of the biological activity of other cytokines, receptor (especially cytokine) activation, deactivation, up-or down regulation, cell growth or differentiation and the like. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the native *mpl* ligand or TPO. The principal antigenic function of a *mpl* ligand or TPO polypeptide is that it binds with an affinity of at least about 10^6 l/mole to an antibody raised against the *mpl* ligand or TPO isolated from aplastic porcine plasma. Ordinarily, the polypeptide binds with an affinity of at least about 10^7 l/mole. Most preferably, the antigenically active *mpl* ligand or TPO polypeptide is a polypeptide that binds to an antibody raised against the *mpl* ligand or TPO having one of the above described effector functions. The antibodies used to define "biological property" are rabbit polyclonal antibodies raised by formulating the *mpl* ligand or TPO isolated from recombinant cell culture or aplastic porcine plasma in Freund's complete adjuvant, subcutaneously injecting the formulation, and

boosting the immune response by intraperitoneal injection of the formulation until the titer of *mpl* ligand or TPO antibody plateaus.

"Thrombopoietic activity" is defined as biological activity characterized by accelerating the proliferation, differentiation and/or maturation of megakaryocytes or megakaryocyte precursors into the platelet producing form of these cells. This activity may be measured in various assays including an in vivo mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-$GPII_bIII_a$) for a human leukemia megakaryoblastic cell line (CMK), and induction of polyploidization in a megakaryoblastic cell line (DAMI).

By the term "low-multiple" in connection with the dosing is meant the administration of multiple doses of therapeutically effective amounts over a short period of time. A low-multiple dose may include 2 to about 6 doses per day, preferably 2-4 doses per day. Thus, the present invention is directed to the mere single administration of a therapeutically effective amount of a thrombopoietin. It has been found that a single administration produces a therapeutic effect equivalent to that realized when a therapeutically effective amount of the same material is administered over the conventional multiple many day regimen suggested and taught by the art.

The term "treatment cycle" as used herein means a course of radiation and/or chemotherapeutic agent administration (treatment phase) in which a mammal or patient is treated with radiation and/or chemotherapeutic agent, generally followed by a period of observation and recovery (recovery phase). The treatment phase may include a single administration of radiation and/or chemotherapeutic agent or multiple administrations, preferably separated by a period of time which is usually chosen so as to minimize discomfort to the mammal or patient and allow recovery of neutrophil and platelet counts to about pretreatment levels. The time period is generally determined by the tolerance of the mammal or patient for the particular radiation and/or chemotherapeutic agent. A typical treatment phase may run 1-10 days, preferably 1-6 or 1-4 days, during which time the radiation or chemotherapeutic agent is administered continuously or portion-wise. A typical recovery phase may run 5-60 days, preferably 14-24 days, during which time the mammal or patient is observed, evaluated and allowed to recover from the treatment. Optionally, more than one treatment cycle may be given, typically 2 to about 6 cycles depending on the particular treatment regimen and the purpose of the treatment.

Detailed Description of the Preferred Embodiments

It has now been discovered that TPO has pharmacokinetic properties which are suprisingly different than the properties of cytokines such as G-CSF and GM-CSF and which allow TPO to be administered prior to and/or concurrently with radiation and/or chemotherapy. Prior and/or concurrent administration of TPO has been found to reduce the depth of the nadir of thrombocytopenia and to shorten the time for platelet titer recovery in patients receiving radiation and/or chemotherapeutic treatment. This difference in properties of TPO is believed to derive from the effect of TPO on early

progenitor cells in the hematopoietic lineage. This effect appears to result in a delay in the appearance of more mature cells in the lineage following administration of TPO, allowing radiation and/or chemotherapy treatments to be given with little or clinically insignificant loss of proliferating cells during cytoreductive treatments. The discovery of these unique properties of TPO is part of the present invention.

This discovery is significant since patients receiving radiation and/or chemotherapy frequently require platelet transfusions. Frequent platelet transfusions may result in alloimmunization. This results in the need for HLA-matched donors and more frequent transfusions. The provision of platelet transfusions is an important and often difficult medical problem. Reducing the need and frequency of such transfusions improves patient care and mitigates complications associated with transfusions, such as blood antigen incompatibilities, lack of suitable platelet donors, contamination of donated materials, e.g. contamination with a virus, etc. Any treatment that prevents or shortens the duration of prolonged thrombocytopenia represents an important medical advance. The present invention reduces the necessity for platelet transfusions in patients experiencing thrombocytopenia.

The method of the invention hastens the recovery of platelet titers to baseline levels and even to substantially elevated levels following radiation and/or chemotherapy. The generation of elevated platelet titers is useful in preparing a patient for subsequent cycles of radiation and/or chemotherapy treatment. A patient entering a subsequent cycle of treatment with elevated platelet levels is better able to withstand the cytoreductive effects of the treatment. The invention, therefore, is effective to increase patient tolerance to a radiation and/or chemotherapeutic regimen relative to the patient tolerance for the regimen without administration of TPO according to the invention.

The method of the invention is also useful in mobilization therapy. In mobilization therapy, the peripheral blood progenitor cells are mobilized from the bone marrow to reduce or eliminate neutropenia and/or thrombocytopenia. In the method of the invention, TPO is administered as a single or low-multiple daily dose to mobilize peripheral blood progenitor cells. Typically, the mammal is a human patient having or at risk for thrombocytpenia, for example, as a result of radiation and/or chemotherapy or liver disease. According to the invention, TPO is administered to the patient prior to or concurrent with radiation or chemotherapy treatment. Of course, TPO may also be administered subsequent to radiation or chemotherapy treatment to restore platelet blood titer in conjunction with the prior or concurrent administration noted above. The TPO may also be administered together with another cytokine, e.g. G-CSF, IL-3, IL-6, GM-CSF, etc. The progenitor cells which are mobilized by the method of the invention may be collected by standard leukapheresis, optionally frozen, and retransplanted into the patient after radiation and/or chemotherapy. The additional cytokine is generally administered in an amount similar to the amount of TPO. For example, in mobilization therapy the TPO might be administered in an amount from about 0.1-10 microgram/kg alone or together with an additional cytokine in a similar amount. For heterologous bone marrow transplants, TPO optionally in combination with another cytokine as discussed above, may be administered to a

mammal including a human patient, for the purpose of mobilizing peripheral blood progenitor cells which may then be harvested by leukapheresis, optionally frozen and transplanted into a mammal having or at risk for thrombocytopenia. The mammal or patient donating the heterologous bone marrow graft (progenitor cells) and the transplant recipient may be tissue-typed according to known procedures. The TPO and other cytokine are generally administered in the amounts discussed above for autologous transplants.

Additionally, it is well known that repeated cycles of radiation and/or chemotherapy result in a cumulative myelosuppression which limits the dose intensity of individual chemotherapeutic agents, particularly when used in combination therapy. Commercially available myeloid growth factors, such as G-CSF, have helped to reduce neutropenia; however cumulative thrombocytopenia remains a problem. The present invention significantly reduces neutropenia during combination chemotherapy thereby increasing the number of treatment cycles a patient will tolerate during chemotherapy. A larger number of treatment cycles or stronger doses of the chemotherapeutic agents improves the cancer kill rate.

The method of the invention also reduces the likelihood of formation of anti-TPO antibodies. Immunogenicity is reduced or eliminated due to less frequent dosing, including single doses, relative to continuous daily dosing known in the art. Dosing is preferably intravenous. However, hybrid regimen in which IV dosing is combined with SC dosing is also contemplated by this invention. For example, it may be desirable to administer an initial IV dose of TPO before or shortly after treatment with radiation and/or chemotherapy in order to reduce the platelet nadir associated with such treatment and to accelerate recovery of platelet titers. This initial dose of TPO might be followed by one or more SC doses of TPO after the treatment to maintain the platelet levels.

An alternative to parenteral or subcutaneous delivery of TPO is aerosol delivery. The pulmonary route of administration is an attractive alternative to intravenous (IV) or subcutaneous (SC) delivery because of the ease of administration and the large surface area of the lung for absorption. However, the barrier to absorption of proteins is formidable and the mechanism of absorption is unclear. Despite the potential limitations, several proteins (e.g. insulin, hGH, BSA, and LHRH) have been delivered successfully via the lungs to target the systemic circulation. See Adjei et al, International J Pharm., 61:135-144, 1990; Colthorpe et al , Pharm. Res., 9:764-768, 1992; Folkesson et al , Acta Physiologica Scandinavia, 139:347-54, 1990; Patton et al, Biotech. Therapeut., 1:213-228, 1989-90; and Niven et al, Pharm. Res., 12:1343-9, 1995. The table shown below indicates proteins which have been tested for delivery via the lung.

Protein*	Molecular Weight (kDa)	Bioavailability+ (%)	Reference
LHRH	1.067	4-18	Adjei, et al, 1990
Insulin	5.7	5.6 (IT)	Colthorpe et al, 1992
Insulin	5.7	57 (aerosol)	Colthorpe et al, 1992
rhG-CSF	18.8	66	Niven et al, 1995
hGH	22.0	35	Patton et al, 1989
BSA	67.0	4.3	Folkesson et al, 1990

* LHRH=Leutinizing hormone releasing hormone, rhG-CSF=recombinant human granulocyte-colony stimulating factor, hGH= human growth hormone, and BSA= bovine serum albumin
+ IT= intratracheal instillation

In an interesting embodiment of this invention, it has been discovered that a TPO, e.g. recombinant human thrombopoietin (rhTPO), can be administered via aerosol inhalation to target the systemic circulation. rhTPO is an about 80 kD glycoprotein. Therapeutic serum concentrations can be achieved by delivering rhTPO to the lungs as a liquid or powder aerosol. Solutions of TPO may be nebulized using conventional nebulizers and administered to a mammal or human patient through the nose or mouth as an aerosol. TPO may also be dried to a powder, e.g. by spray-drying, and administered using a conventional dry powder inhaler. A higher dose of rhTPO is required to achieve a similar therapeutic effect when given as an aerosol as compared to IV. Generally, the dose by aerosol should be about 100-fold higher for aerosol administration as compared to IV administration. A suitable dose range for aerosol administration is about 5-1000 microgram/kg, preferably 50-750 microgram/kg, as a single inhalation dose or as multiple inhalations on a single day or on multiple days, preferably 2-10 or 2-6, sequential or non-sequential days.

The method of the invention may be used with any radiation and/or chemotherapy regimen in which a mammal or human patient is having or is at risk of thrombocytopenia. The method may be used with conventional chemotherapeutic agents used in conventional amounts including, but not limited to asparaginase, bleomycin, calcium leucovorin, carmustine, carboplatin, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, doxorubicin, epirubicin, etoposide, fluorouracil, fluoxymesterone, flutamide, hexamethylmelamine, hydroxyurea, ifosfamide, leuprolide, levamisole, leuprolide depot, lomustine, mechlorethamine, melphalan, mercaptopurine, methotrexate, methyl-CCNU (semustine), methylprednisolone, mitomycin C, mitoxantrone, prednisolone, prednisone, procarbazine, streptozocin, tamoxifen, thioguanine, triethylene-thiophosphoramide, vinblastine, vincristine, and combinations thereof. These compounds may, optionally, be given with a known uroprotecting compound such as mesna, etc. where appropriate and indicated. Mesna is commercially available and is routinely give to counteract the urinary tract irritation and hemorragic cystitis due to chemotherapeutic agent metabolites, e.g. ifosfamide

metabolites. Typically, the chemotherapeutic agents are given in combination to maximize tumor cell kill with minimal or at least acceptable toxicity to the mammal or patient. The method of the invention further reduces this toxicity. Suitable non-limiting chemotherapy regimen with which the method of the invention may be used are listed below using conventional acronyms and indicating specific tumors/cancers for which the regimen is contemplated. The chemotherapeutic agents may be administered in conventional amounts and according to conventional treatment times and regimen. See, for example, "The Cerenex Handbook", Robert S. Benjamin, Ed., Cerenex Pharmaceuticals, Research Triangle Park, N.C. (1993); "Combination Cancer Chemotherapy Regimens", Roger W. Anderson and William J. Dana, Eds., Laderley Laboratories (1991). Any cytoreductive regimen which induces thrombocytopenia is considered to within the scope of the invention, however.

Breast Cancer

CAF - cyclophosphamide, doxorubicin, fluorouracil

CFM - cyclophosphamide, doxorubicin, mitoxantrone

CFPT - cyclophosphamide, fluorouracil, prednisolone, tamoxifen

CMF - cyclophosphamide, methotrexate, fluorouracil

CMFP - cyclophosphamide, methotrexate, fluorouracil, prednisolone

CMFVP - cyclophosphamide, methotrexate, fluorouracil, vincristine, prednisolone

FAC - fluorouracil, doxorubicin, cyclophosphamide

IMF - ifosfamide, methotrexate, fluorouracil, mesna

VATH - vinblastine, doxorubicin, thiotepa, fluoxymesterone

CEP - cyclophosphamide, etoposide, cisplatin

ICE - ifosfamide, cyclophosphamide, etoposide

AC - doxorubicin, cyclophosphamide

FLAC - fluorouracil, calcium leucovorin, doxorubicin, cyclophosphamide

Colon Cancer

F-CL - Fluorouracil, calcium leucovorin

FLe - levamisole, fluorouracil

FMV - fluorouracil, methyl-CCNU, vincristine

Gastric Cancer

FAM - fluorouracil, doxorubicin, mitomycin C

FAME - fluorouracil, doxorubicin, methyl-CCNU

FCE - fluorouracil, cisplatin, etoposide

Genitourinary Cancer

CAP - cisplatin, doxorubicin, cyclophosphamide

CISCA - cyclophosphamide, doxorubicin, cisplatin

CVEB - cisplatin, vinblastine, etoposide, bleomycin

FL - flutamide, leuprolide acetate or flutamide, leuprolide acetate depot

L-VAM - leuprolide acetate, vinblastine, doxorubicin, mitomycin C

MVAC - methotrexate, vinblastine, doxorubicin, cisplatin

VAB - vinblastine, dactinomycin, bleomycin, cisplatin, cyclophosphamide

VB - vinblastine, methotrexate

VBP - vinblastine, bleomycin, cisplatin Gestational Trophoblastic Disease

DMC - dactinomycin, methotrexate, cyclophosphamide

Head and Neck Cancer

CF - cisplatin, fluorouracil

CFL - cisplatin, fluorouracil, calcium leucovorin

COB - cisplatin, vincristine, bleomycin

MAP - mitomycin C, doxorubicin, cisplatin

MBC - methotrexate, bleomycin, cisplatin

MF - methotrexate, fluorouracil, calcium leucovorin

Leukemias

Acute Lymphocytic Leukemia (A.L.L.)

DVP - daunorubicin, vincristine, prednisone

MM - mercaptopurine, methotrexate

AVDP - asparaginase, vincristine, daunorubicin, prednisone

Acute Myelogenous Leukemia (A.M.L.)

AA - cytarabine, doxorubicin

COAP - cyclophosphamide, vincristine, cytarabine, prednisone

MV - mitoxantrone, etoposide

Acute Non-Lymphocytic Leukemia (A.N.L.L.)

DCT - daunorubicin, cytarabine, thioguanine

MC - mitoxantrone, cytarabine

CD - cytarabine, daunorubicin

TC - thioguanine, cytarabine

Chronic Lymphatic Leukemia (C.L.L.)

CVP - cyclophosphamide, vincristine, prednisone

Lung Cancer

Small Cell

COPE - cyclophosphamide, cisplatin, etoposide, vincristine

CV - cisplatin, etoposide

VAC - vincristine, doxorubicin, cyclophosphamide

VC - etoposide, carboplatin

ICE - ifosfamide, cyclophosphamide, etoposide

CEP - cyclophosphamide, etoposide, cisplatin

Non-small Cell

BACON - bleomycin, doxorubicin, lomustine, vincristine, mechlorethamine

CAMP - cyclophosphamide, doxorubicin, methotrexate, procarbazine

CAP - cyclophosphamide, doxorubicin, cisplatin

CV - cisplatin, etoposide

CVI - cisplatin, etoposide, ifosfamide, mesna

FAM - fluorouracil, doxorubicin, mitomycin C

FOMi/CAP - fluorouracil, vincristine, mitomycin C, cyclophosphamide, doxorubicin, cisplatin

MACC - methotrexate, doxorubicin, cyclophosphamide, lomustine

ICE - ifosfamide, cyclophosphamide, etoposide

CEP - cyclophosphamide, etoposide, cisplatin

Lymphoma

Hodgkin's

ABVD - doxorubicin, bleomycin, vincristine, dacarbazine

B-CAVe - bleomycin, lomustine, doxorubicin, vinblastine

B-DOPA - bleomycin, dacarbazine, vincristine, prednisone, doxorubicin

CVPP - lomustine, vinblastine, procarbazine, prednisone

MOPP - mechlorethamine, vincristine, procarbazine, prednisone

MVPP - mechlorethamine, vinblastine, procarbazine, prednisone

NOVP - mitoxantrone, vinblastine, prednisone, vincristine

Non-Hodgkin's

ASHAP - doxorubicin, cisplatin, cytarabine, methylprednisolone

BACOP - bleomycin, doxorubicin, cyclophosphamide, vincristine, prednisone

CHOP - cyclophosphamide, doxorubicin, vincristine, prednisone

CHOP-Bleo - cyclophosphamide, doxorubicin, vincristine, prednisone , bleomycin

COMLA - cyclophosphamide, vincristine, methotrexate, calcium leucovorin, cytarabine

COP - cyclophosphamide, vincristine, prednisone

COPP - cyclophosphamide, vincristine, procarbazine, prednisone

CVP - cyclophosphamide, vincristine, prednisone

E-SHAP - etoposide, cisplatin, cytarabine, methylprednisolone

IMVP-16 - ifosfamide, mesna, methotrexate, etoposide

m-BACOD - bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone, methotrexate, calcium leucovorin

m-BACOS - doxorubicin, vincristine, bleomycin, cyclophosphamide, methotrexate, calcium leucovorin

MINE - mesna, ifosfamide, mitoxantrone, etoposide

OPEN - etoposide, mitoxantrone, vincristine, prednisone

Pro-MACE - prednisone, methotrexate, calcium leucovorin, doxorubicin, cyclophosphamide, etoposide

AI - doxorubicin, ifosfamide

AC - doxorubicin, cyclophosphamide

ICE - ifosfamide, cyclophosphamide, etoposide

CEP - cyclophosphamide, etoposide, cisplatin

Malignant Melanoma

BHD - carmustine, hydroxyurea, dacarbazine

DTIC-ACTD - dacarbazine, dactinomycin

VBC - vinblastine, bleomycin, cisplatin

VDP - vinblastine, dacarbazine, cisplatin

Multiple Myeloma

AC - doxorubicin, carmustine

BCP - carmustine, cyclophosphamide, prednisone

MeCP - methyl-CCNU, cyclophosphamide, prednisone

MP - melphalan, prednisone

M-2 - vincristine, carmustine, cyclophosphamide, melphalan, prednisone

VAD - vincristine, doxorubicin, dexamethasone

VBAP - vincristine, carmustine, doxorubicin, prednisone

VCAP - vincristine, cyclophosphamide, doxorubicin, prednisone

Ovarian Cancer

Epithelial

C - carboplatin

AP - doxorubicin, cisplatin

CDC - carboplatin, doxorubicin, cyclophosphamide

CHAD - cyclophosphamide, doxorubicin, cisplatin, hexamethylmelamine

CHAP - cyclophosphamide, hexamethylmelamine, doxorubicin, cisplatin

CP - cyclophosphamide, cisplatin

PAC - cisplatin, doxorubicin, cyclophosphamide

AC - doxorubicin, cyclophosphamide

ICE - ifosfamide, cyclophosphamide, etoposide

CEP - cyclophosphamide, etoposide, cisplatin

Germ Cell

VAC - vincristine, dactinomycin, cyclophosphamide

Endometrial Cancer

C - carboplatin

AC - doxorubicin, cyclophosphamide

Pancreatic Cancer

FMS - fluorouracil, mitomycin C, streptozocin

SD - streptozocin, doxorubicin

Pediatric Tumors

A.L.L.

DVP - daunorubicin, vincristine, prednisone

VAP - daunorubicin, asparaginase, prednisone

CT - cytarabine, thioguanine

DCPM - daunorubicin, cytarabine, prednisolone, mercaptopurine

A.N.L.L.

DC - daunorubicin, cytarabine

Bony sarcoma

AC - doxorubicin, cisplatin

HDMTX - methotrexate, calcium leucovorin

T-2 - dactinomycin, doxorubicin, vincristine, cyclophosphamide

Hodgkin's disease

ACOPP - doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone

MOPP - mechlorethamine, vincristine, procarbazine, prednisone

Soft Tissue Sarcoma

VAC - vincristine, dactinomycin, cyclophosphamide

Sarcoma

Bony Sarcoma

AC - doxorubicin, cisplatin

CYVADIC - cyclophosphamide, vincristine, doxorubicin, dacarbazine

HDMTX - methotrexate, calcium leucovorin

IMAC - ifosfamide, mesna, doxorubicin, cisplatin

Soft Tissue

CYADIC - cyclophosphamide, doxorubicin, dacarbazine

CYVADIC - cyclophosphamide, vincristine, doxorubicin, dacarbazine

ID - ifosfamide, mesna, doxorubicin

VAC - vincristine, dactinomycin, cyclophosphamide

AI - ifosfamide, doxorubicin

MAID - mesna, doxorubicin, ifosfamide, dacarbazine

Selected regimens particularly associated with thrombocytopenia which may be treated with the method of the invention include:

	Regimens	Malignancy
•	carboplatin/paclitaxel	Ovary NSCLC
•	FUDR/leucovorin/doxorubicin/cisplatin	HBV+/HCV+ Hepatoma
•	cisplatin/alpha interferon/5-FU/ doxorubicin	HBV+/HCV+ Hepatoma
•	ifosfamide/mesna/carboplatin/etoposide	NSCLC
•	cisplatin/ifosfamide/mesna/etoposide	Testicle
•	High-dose carboplatin/etoposide	Testicle (salvage)
•	methotrexate/vinblastine/doxorubicin/ cisplatin	Bladder
•	BCNU (carmustine)	Glioblastoma
•	procarbazine/CCNU/vincristine	Brain
•	cyclophaphamide/etoposide +/- cisplatin or carboplatin	Salvage Hodgkin's disease, breast, ovary, non-Hodgkin's lymphoma, head and neck, lung, myeloma, sarcoma
•	dexamethasone/HIDAC/cisplatin	salvage non-Hodgkin's lymphoma
•	mesna/ifosfamide/doxorubicin/$\pm$ DTIC	Soft tissue sarcoma
•	cyclophophamide/vincristine/DTIC doxorubicin	Soft tissue sarcoma

The single or low multiple dose of the invention may be given prior to the first treatment time of radiation and/or chemotherapeutic agent in a treatment cycle, during or concurrent with a treatment time in a treatment cycle or following one or more individual treatment times of radiation or chemotherapeutic agent in a treatment cycle. For example, a cycle may constitute a single treatment time of radiation or chemotherapeutic agent. In the invention, the single or low-multiple dose of TPO would be administered before, during or after this treatment time. Alternatively, the cycle may constitute multiple treatment times, for example 2-10 or more treatment times, of radiation or chemotherapeutic agent. Here, the invention contemplates administering TPO before, during or after any one treatment time or before, during or after each individual treatment time. For example, the cycle may constitute three treatments with a chemotherapeutic agent. In the method of the invention, TPO might be administered before each of the three treatment times or might be administered after each of the three treatment times. Of course, the invention also includes administration of a single

daily dose of TPO before the first treatment time of the chemotherapeutic agent in the cycle and after the last treatment time in the cycle.

In a preferred embodiment, the mammal receives at least one treatment cycle of radiation and/or chemotherapeutic agent, where the treatment cycle has a first treatment time T_0 and a last treatment time T_F for administering radiation and/or chemotherapeutic agent. The dose of TPO is preferably administered at T_0 plus or minus 24 hours, more preferably T_0 plus or minus 10 hours, still more preferably T_0 plus or minus 6 hours, and most preferably T_0 plus or minus 2 hours.

In alternative embodiments, the dose is administered at T_0 or prior to T_0, but not more than seven days prior to T_0, preferably not more than one day prior to T_0. For single dose cycles, $T_0 = T_F$. In another preferred embodiment, the dose is administered prior to T_F, but not more than seven days prior to T_F. As noted above, TPO may also be administered after T_F. When a second dose of TPO is administered after T_F, the dose is preferably administered not more than 24 hours after T_F. The mammal or patient may, of course, receive multiple treatment cycles, generally 2-6 cycles, but as many cycles as is medically necessary to reduce the size of or to completely irradicate a cancer or tumor. In some treat regimen, a tumor is reduced in size relative to the size of the tumor prior to radiation and/or chemotherapy treatment, and then surgery is utilized to remove the remaining malignant tissue of the tumor. The method of the invention may be used in these regimen as well.

The invention also includes co-administering a therapeutically effective amount of a cytokine, a colony stimulating factor and an interleukin, generally after administration of the TPO dose, preferably after administration of the last TPO dose in a treatment cycle. The cytokine is preferably KL, LIF, G-CSF, GM-CSF, M-CSF, EPO, FLT-3, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 or IL-11, in particular, G-CSF or GM-CSF.

It has been found in accord with the present invention that the single or low multiple administration regimen of the present invention is effective at daily dosage rates on the order of about 0.1 to 50, preferably about 0.1 to 10, more preferably about 1 to 5, or preferably about 1 to 3 microgram/kg body weight of the patient. In single dosing, preferred would be the total administration of about 2 ±1.5 microgram/kg of body weight. In low-multiple dosing, preferred would be the administration of from about 0.5 to 1.5 microgram/kg body weight per dose. The above dosages are predicated on preferred intravenous administration. In administration via the subcutaneous route, the total amount administered would be in the range of about one to three times the amount administered via the intravenous route, preferably about two times. Further, the doses for administration via the lung are higher as noted above. Specific therapeutically effective dosages for individual patients may be determined by conventional methods.

The method of the invention also preferably provides a dose of TPO sufficient to maintain a blood TPO level in the mammal of 35×10^{-12} M or greater during the radiation and/or chemotherapy

treatment cycle. Preferably, the dose is sufficient to maintain a blood TPO level of 100×10^{-12} M or greater, more preferably about 35×10^{-12} M to about 3500×10^{-12} M during the treatment cycle.

The optimal dosage rate and regimen will be determined by the attending physician taking into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors.

It will be understood that although a single daily administration of a thrombopoietin to a patient has been found to be therapeutically effective for the treatment of thrombocytopenia, it can be appreciated that a low-multiple (daily) regimen may be employed. It has been found that a single dose stimulates the onset of therapeutic response, and although multiple dosing is contemplated herein, termination of dosing after a single or low-multiple administration is independent of therapeutic response.

The biologically active thrombopoietin materials of the present invention can be administered, in accord herewith, in various routes including via the nose or lung, subcutaneously, and preferably intravenously. In all events, depending upon the route of administration, the biologically active thrombopoietin materials of the present invention are preferably administered in combination with an appropriate pharmaceutically acceptable carrier or excipient. When administered systemically, the therapeutic composition should be pyrogen-free and in a parenterally acceptable solution having due regard for physiological pH isotonicity and stability. These conditions are generally well known and accepted to those of skill in the appropriate art.

Briefly, dosage formulations of the materials of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients and/or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed and include buffers such as phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight peptides such as polyarginine, proteins such as serum albumen, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid or arginine; monosaccharides, disaccharides and other carbohydrates including cellulose or its derivatives, glucose, mannose or dextrins; chelating agents such as EDTA; sugar alcohol such as mannitol or sorbitol; counter-ions such as sodium; and/or non-ionic surfactants such as TWEEN, PLURONICS or polyethylene glycol.

The biologically active thrombopoietin materials hereof can be administered as the free acid or base form or as a pharmaceutically acceptable salt and are compounded with a physiologically acceptable vehicle, carrier, excipient, binder, preservative, stabilizer, flavoring agent, etc. as called for by accepted pharmaceutical practice.

Sterile compositions for injection can be formulated according to conventional pharmaceutical or pharmacological practice. For example, dissolution or suspension of the active material in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Again, buffers, preservatives, anti-oxidants and the like can be incorporated according to accepted pharmaceutical practice. The biologically active thrombopoietin materials of the present invention may be employed alone or administered in combination with other cytokines, hematapoietins, interleukins, growth factors, or antibodies in the treatment of the above identified disorders and conditions marked by thrombocytopenia. Thus, the present active materials may be employed in combination with other protein or peptide having thrombopoietic activity including: G-CSF, GM-CSF, LIF, M-CSF, IL-2, IL-3, erythropoietin (EPO), Kit ligand, IL-6, IL-11, FLT-3 ligand, and so forth.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (*e.g.* poly(2-hydroxyethyl-methacrylate) as described by Langer *et al. J. Biomed. Mater. Res., 15*:167-277 (1981) and Langer, *Chem. Tec., 12*:98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,779,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.Biopolymers, 22*:547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer *et al., supra*), degradable lactic acid-glycolic acid copolymers such as the LUPROM DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release thrombopoietic protein compositions also include liposomally entrapped megakaryocytopoietic protein. Liposomes containing megakaryocytopoietic protein are prepared by methods knew *per se*: DE, 3,218,121; Epstein *et al.Proc. Natl. Acad. Sci. USA, 82*:3688-3698 (1985); Hwang *et al.Proc. Natl. Acad. Sci. USA, 77*:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms)

unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal megakaryocytopoietic protein therapy.

A type of covalent modification of TPO or *mpl* ligand comprises linking the TPO polypeptide to one of a variety of nonproteinaceous polymers, *e.g.* polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. TPO polypeptides covalently linked to the forgoing polymers are referred to herein as pegylated TPO.

It will be appreciated that some screening of the recovered TPO variant will be needed to select the optimal variant for binding to a *mpl* and having the immunological and/or biological activity defined above. One can screen for stability in recombinant cell culture or in plasma (*e.g.*, against proteolytic cleavage), high affinity to a *mpl* member, oxidative stability, ability to be secreted in elevated yields, and the like. For example, a change in the immunological character of the TPO polypeptide, such as affinity for a given antibody, is measured by a competitive-type immunoassay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, or susceptibility to proteolytic degradation are assayed by methods well known in the art.

Methods of Making

Isolation of the Human mpl Ligand (TPO) Gene

Human genomic DNA clones of the TPO gene were isolated by screening a human genomic library in 8-Gem12 with pR45, under low stringency conditions or under high stringency conditions with a fragment corresponding to the 3N half of human cDNA coding for the *mpl* ligand. Two overlapping lambda clones spanning 35 kb were isolated. Two overlapping fragments (BamH1 and EcoRI) containing the entire TPO gene were subcloned and sequenced.

The structure of the human gene is composed of 6 exons within 7 kb of genomic DNA. The boundaries of all exon/intron junctions are consistent with the consensus motif established for mammalian genes (Shapiro, M.B. *et al., Nucl. Acids. Res. 15*:7155 (1987)). Exon 1 and exon 2 contain 5N untranslated sequence and the initial four amino acids of the signal peptide. The remainder of the secretory signal and the first 26 amino acids of the mature protein are encoded within exon 3. The entire carboxyl domain and 3N untranslated as well as ~50 amino acids of the erthropoietin-like domain are encoded within exon 6. The four amino acids involved in the deletion observed within hML-2 (hTPO-2) are encoded at the 5N end of exon 6.

Analysis of human genomic DNA by Southern blot indicated the gene for TPO is present in a single copy. The chromosomal location of the gene was determined by fluorescent *in situ* hybridization (FISH) which mapped to chromosome 3q27-28.

Expression and Purification of TPO from 293 Cells

Preparation and purification of ML or TPO from 293 cells is described in detail in Example 1. Briefly, cDNA corresponding to the TPO entire open reading frame was obtained by PCR using pRK5-h*mpl* I. The PCR product was purified and cloned between the restriction sites ClaI and XbaI of the plasmid pRK5tkneo.ORF (a vector coding for the entire open reading frame).

A second vector coding for the EPO homologous domain was generated the same but using different PCR primers to obtain the final construct called pRK5-tkneoEPO-D.

These two constructs were transfected into human embryonic kidney cells by the $CaPO_4$ method and neomycin resistant clones were selected and allowed to grow to confluency. Expression of ML_{153} or ML_{332} in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay.

Purification of $rhML_{332}$ was conducted as described in Example 1. Briefly, 293-$rhML_{332}$ conditioned media was applied to a BLUE-SEPHAROSE (Pharmacia) column that was subsequently washed with a buffer containing 2M urea. the column was eluted with a buffer containing 2M urea and 1M NaCl. The BLUE-SEPHAROSE elution pool was then directly applied to a WGA-SEPHAROSE column, washed with 10 column volumes of buffer containing 2M urea and 1M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-SEPHAROSE eluate was applied to a C4-HPLC column (Synchrom, Inc.) and eluted with a discontinuous propanol gradient. By SDS-PAGE the purified 293-$fhML_{332}$ migrates as a broad band in the 68-80 kDa region of the gel.

Purification of $rhML_{153}$ was also conducted as described in Example 1. Briefly, 293-$rhML_{153}$ conditioned media was resolved on BLUE-SEPHAROSE as described for $rhML_{332}$. The BLUE-SEPHAROSE eluate was applied directly to a *mpl*-affinity column as described above. $RhML_{153}$ eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC column run under the same conditions used for $rhML_{332}$. By SDS-PAGE the purified $rhML_{153}$ resolves into 20 major and 2 minor bands with Mr of ~18,000-22,000.

Expression and Purification of TPO from Chinese Hamster Ovary (CHO) Cells

The expression vectors used to transfect CHO cells are designated: pSVI5.ID.LL.MLORF (full length of TPO_{332}), and pSVI5.ID.LL.MLEPO-D (truncated or TPO_{153}).

cDNA corresponding to the entire open reading frame of TPO was obtained by PCR. The PCR product was purified and cloned between two restriction sites (ClaI and SalI) of the plasmid pSVI5.ID.LL to obtain the vector pSVI5.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using a different reverse primer (EPOD.Sal). The final construct for the vector coding for the EPO homologous domain of TPO is called pSVI5.ID.LL.MLEPO-D.

These two constructs were linearized with NotI and transfected into Chinese Hamster Ovary cells (CHO-DP12 cells, EP 307,247 published 15 March 1989) by electroporation. 10^7 cells were electroporated in a BRL electroporation apparatus (350 Volts, 330 mF, low capacitance) in the presence of 10, 25 or 50 mg of DNA as described (Andreason, G.L. *J.Tissue Cult. Meth., 15*:56 (1993)). The day following transfection, cells were split in DHFR selective media (High glucose DMEM-F12 50:50 without glycine, 2mM glutamine, 2-5% dialyzed fetal calf serum). 10 to 15 days later individual colonies were transferred to 96 well plates and allowed to grow to confluency. Expression of ML_{153} or ML_{332} in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay.

The process for purifying and isolating TPO from harvested CHO cell culture fluid is described in Example 2. Briefly, harvested cell culture fluid (HCCF) is applied to a BLUE-SEPHAROSE column (Pharmacia) at a ratio of approximately 100L of HCCF per liter of resin. The column is then washed with 3 to 5 column volumes of buffer followed by 3 to 5 column volumes of a buffer containing 2.0M urea. TPO is then eluted with 3 to 5 column volumes of buffer containing both 2.0M urea and 1.0M NaCl.

The BLUE-SEPHAROSE eluate pool containing TPO is then applied to a wheat germ lectin SEPHAROSE column (Pharmacia) equilibrated in the BLUE-SEPHAROSE eluting buffer at a ratio of from 8 to 16 ml of BLUE-SEPHAROSE eluate per ml of resin. The column is then washed with 2 to 3 column volumes of equilibration buffer. TPO is then eluted with 2 to 5 column volumes of a buffer containing 2.0M urea and 0.5M N-acetyl-D-glucosamine.

The wheat germ lectin eluate containing TPO is then acidified and $C_{12}E_8$ is added to a final concentration of 0.04%. The resulting pool is applied to a C4 reversed phase column equilibrated in 01% TFA, 0.04% $C_{12}E_8$ at a load of approximately 0.2 to 0.5 mg protein per ml of resin.

The protein is eluted in a two phase linear gradient of acetonitrile containing 0.1% TFA and 0.04% $C_{12}E_8$ and a pool is made on the basis of SDS-PAGE.

The C4 Pool is then diluted and diafiltered versus approximately 6 volumes of buffer on an AMICON YM or like ultrafiltration membrane having a 10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be then directly processed or further concentrated by ultrafiltration. The diafiltrate/concentrate is usually adjusted to a final concentration of 0.01% TWEEN-80.

All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the calculated column volume is then applied to a SEPHACRYL S-300 HR column (Pharmacia) equilibrated in a buffer containing 0.01% TWEEN-80 and chromatographed. The TPO containing fractions which are free of aggregate and proteolytic degradation products are then pooled on the basis of SDS-PAGE. The resulting pool is filtered and stored at 2-8°C.

Methods for Transforming and Inducing TPO Synthesis in a Microorganism and Isolating, Purifying and Refolding TPO Made Therein

Construction of *E. coli* TPO expression vectors is described in detail in Example 3. Briefly, plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 were all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for high level translation initiation and rapid purification. The plasmids pMP210-1, -T8, -21, 22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy-terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in *E. coli* upon induction of the tryptophan promoter (Yansure, D. G. *et al.*, *Methods in Enzymology, 185*:54-60 (Goeddel, D.V., Ed.) Academic Press, San Diego (1990)). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids. The above TPO expression plasmids were used to transform the *E. coli* using the $CaCl_2$ heat shock method (Mandel, M. *et al.*, *J. Mol. Biol, 53*:159-162, (1970)) and other procedures described in Example 3. Briefly, the transformed cells were grown first at 37°C until the optical density (600 nm) of the culture reached approximately 2-3. The culture was then diluted and, after growth with aeration, acid was added. The culture was then allowed to continue growing with aeration for another 15 hours after which time the cells were harvested by centrifugation.

The isolation, purification and refolding procedures given below for production of biologically active, refolded human TPO or fragments thereof is described in Example 4 can be applied for the recovery of any TPO variant including N and C terminal extended forms. Other procedures suitable for refolding recombinant or synthetic TPO can be found in the following patents: Builder *et al.*, US 4,511,502; Jones *et al.*, US 4,512,922; Olson, US 4,518,526 and Builder *et al.*, US 4,620,948; for a general description of the recovery and refolding process for a variety of recombinant proteins expressed in an insoluble form in *E. coli* .

Methods for Measurement of Thrombopoietic Activity

Thrombopoietic activity may be measured in various assays including the Ba/F3 *mpl* ligand assay. An *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-$GPII_bIII_a$) for a human leukemia megakaryoblastic cell line (CMK) (see Sato *et al.*, *Brit. J. Heamatol., 72*:184-190 (1989)) and induction of polyploidization in a megakaryoblastic cell line (DAMI) (see Ogura *et al.*, *Blood, 72*(1):49-60 (1988)). Maturation of megakaryocytes from immature, largely non-DNA synthesizing cells, to morphologically identifiable megakaryocytes involves a process that includes appearance of

cytoplasmic organelles, acquisition of membrane antigens ($GPII_bIII_a$), endoreplication and release of platelets as described in the background. A lineage specific promoter (*i.e.* the *mpl* ligand) of megakaryocyte maturation would be expected to induce at least some of these changes in immature megakaryocytes leading to platelet release and alleviation of thrombocytopenia. Thus, assays were designed to measure the emergence of these parameters in immature megakaryocyte cell lines, i.e., CMK and DAMI cells. The CMK assay measures the appearance of a specific platelet marker, $GPII_bIII_a$, and platelet shedding. The DAMI assay measures endoreplication since increases in ploidy are hallmarks of mature megakaryocytes. Recognizable megakaryocytes have ploidy values of 2N, 4N, 8N, 16N, 32N, etc. Finally, the *in vivo* mouse platelet rebound assay is useful in demonstrating that administration of the test compound (here the *mpl* ligand) results in elevation of platelet numbers.

Two additional *in vitro* assays have been developed to measure TPO activity. The first is a kinase receptor activation (KIRA) ELISA in which CHO cells are transfected with a *mpl*-Rse chimera and tyrosine phosphorylation of Rse is measured by ELISA after exposure of the *mpl* portion of the chimera to *mpl* ligand. The second is a receptor based ELISA in which ELISA plate coated rabbit anti-human IgG captures human chimeric receptor *mpl*-IgG which binds the *mpl* ligand being assayed. A biotinylated rabbit polyclonal antibody to *mpl* ligand (TPO_{155}) is used to detect bound *mpl* ligand which is measured using streptavidin-peroxidase.

Therapeutic Use of Thrombopoietin Materials

The biologically active thrombopoietic protein (TPO) may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia (*e.g.* aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the compounds of this invention as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia. Additionally, these megakaryocytopoietic proteins may be useful in treating myeloproliferative thrombocytotic diseases as well as thrombocytosis from inflammatory conditions and in iron deficiency.

The method of the invention is also useful to treat mammals or human patients which have suffered from exposure to ionizing radiation sufficient to cause thrombocytopenia, for example, persons exposed to nuclear accidents such as the well-known accident which occurred at Chernobyl. TPO is well tolerated by patients and this may justify the rapid administration of TPO within a few hours after a nuclear accident to all persons affected by radiation. As noted in more detail below, the TPO responsiveness of progenitor cells appears to be very large shortly after exposure of a person to

radiation and/or chemotherapy. the method of the invention may also be used as a radioprotective procedure by administering TPO prophylactically to a person who will be exposed to ionizing radiation. For example, an emergency worker may be required to enter highly contaminated areas in cases of a major nuclear accident. Administration of a prophylactic dose of TPO prior to exposure according to the dosing methods of the present invention will ensure that the worker has high levels of early multilineage progenitor cells in order to reduce the degree of thrombocytopenia induced by the exposure to radiation.

Preferred uses of the thrombocytopoietic protein (TPO) of this invention are in: myelotoxic chemotherapy for treatment of leukemia or solid tumors, myeloablative chemotherapy for autologous or allogeneic bone marrow transplant, myelodysplasia, idiopathic aplastic anemia, congenital thrombocytopenia, and immune thrombocytopenia.

Still other disorders usefully treated with the thrombopoietin proteins of this invention include defects or damage to platelets resulting from drugs, poisoning or activation on artificial surfaces. In these cases, the instant compounds may be employed to stimulate "shedding" or new "undamaged" platelets.

Examples

EXAMPLE 1 - Expression and Purification of TPO from 293 Cells

Preparation of 293 Cell Expression Vectors

A cDNA corresponding to the TPO entire open reading frame was obtained by PCR using the following oligonucleotides as primers:

TABLE 1 293 PCR Primers
Cla.FL.F:5N ATC GAT ATC GAT CAG CCA GAC ACC CCG GCC AG 3N (SEQ ID NO:1)
hmpII-R: 5N GCT AGC TCT AGA CAG GGA AGG GAG CTG TAC ATG AGA 3N (SEQ ID NO:2)

prk5-H*mpl* was used as a template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C. the PCR product was purified and cloned between the restriction sites ClaI and XbaI of the plasmid pRK5tkneo,

a pRK5 derived vector modified to express a neomycin resistance gene under the control of the thymidine kinase promote, to obtain the vector pRK5tkneo.ORF. A second construct corresponding to the epo homologous domain was generated the same way but using Cla.FL.F as forward primer and the following reverse primer:

Arg. STOP.Xba: 5NTCT AGA TCT AGA TCA CCT GAC GCA GAG GGT GGA CC 3N (SEQ ID NO: 3)

The final construct is called pRK5-tkneoEPO-D. The sequence of both constructs was verified.

Transfection of Human Embryonic Kidney cells

These 2 constructs were transfected into human embryonic kidney cells by the $CaPO_4$ method. 24 hours after transfection selection of neomycin resistant clones was started in the presence of 0.4 mg/ml G418. 10 to 15 days later individual colonies were transferred to 96 well plates and allowed to grow to confluency. Expression of ML_{153} or ML_{332} (TPO153 or TPO 332) in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay.

Purification of $rhML_{332}$

392-$rhML_{332}$ conditioned media was applied to a BLUE-SEPHAROSE (Pharmacia) column that was equilibrated in 10 mM sodium phosphate pH 7.4 (buffer A). The column was subsequently washed with 10 column volumes each of buffer A and buffer A containing 2M urea. The column was then eluted with buffer A containing 2M urea and 1M NaCl. The BLUE-SEPHAROSE elution pool was then directly applied to a WGA-SEPHAROSE column equilibrated in buffer A. The WGA-SEPHAROSE column was then washed with 10 column volumes of buffer A containing 2M urea and 1 M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-SEPHAROSE eluate was applied to a C4-HPLC column (Synchrom, Inc.) equilibrated in 0.1% TFA. The C4-HPLC column was eluted with discontinuous propanol gradient (0-25%, 25-35%, 35-70%). $rhML_{332}$ was found to elute in the 28-30% propanol region of the gradient. by SDS-PAGE the purified $rhML_{332}$ migrates as a broad band in the 68-8- kDa region of the gel.

Purification of $rhML_{153}$

392-$rhML_{153}$ conditioned media was resolved on BLUE-SEPHAROSE as described for $rhML_{332}$. The BLUE-SEPHAROSE eluate was applied directly to a *mpl*-affinity column as described above. $RhML_{153}$ eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC

column run under the same conditions as described for $rhML_{332}$. By SDS-PAGE the purified $rhML_{153}$ resolves into 2 major and 2 minor bands with Mr of ~18,000-21,000.

EXAMPLE 2 - Expression and Purification of TPO from CHO

1. Description of CHO Expression Vectors

The expression vectors used in the electroporation protocols described below have been designated:

pSV15.ID.LL.MLORF (full length or hTPO332), and

pSV15.ID.LL.MLEPO-D (truncated or $hTPO_{153}$).

2. Preparation of CHO Expression Vectors

A cDNA corresponding to the hTPO entire open reading frame was obtained by PCR using the oligonucleotide primes of the following Table.

CHO Expression Vector PCR Primers

Cla.FL.F2 5' ATC GAT ATC GAT AGC CAG ACA CCC CGG CCA G 3' (SEQ ID NO:4)
ORF.Sal 5' AGT CGA CGT CGA CGT CGG CAG TGT CTG AGA ACC 3' (SEQ ID NO:5)

PRK5-*hmpl* I was used as template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C. The PCR product was purified and cloned between the restriction sites ClaI and SalI of the plasmid pSV15.ID.LL to obtain the vector pSV15.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using Cla.FL.F2 as forward primer and the following reverse primer: EPOD.Sal 5' AGT CGA CGT CGA CTC ACC TGA CGC AGA GGG TGG ACC 3' (SEQ ID NO:6). The final construct is called pSV15.ID.LL.MLEPO-D. The sequence of both constructs was verified.

In essence, the coding sequences for the full length and truncated ligand were introduced into the multiple cloning site of the CHO expression vector pSV15.ID.LL. This vector contains the SV40 early promoter/enhancer region, a modified splice unit containing the mouse DHFR cDNA, a multiple cloning site for the introduction of the gene of interest (in this case the TPO sequences described) an

SV40 polyadenylation signal and origin of replication and the beta-lactamase gene for plasmid selection and amplification in bacteria.

3. Methodology for Establishing Stable CHO Cell Lines Expressing Recombinant Human TPO_{332} and TPO_{153}

a. Description of CHO parent cell line

The host CHO (Chinese Hamster Ovary) cell line used for the expression of the TPO molecules described herein is known as CHO-DP12 (see EP 307,247 published 15 March 1989). This mammalian cell line was clonally selected from a transfection of the parent line (CHO-K1 DUX-B11 (DHFR-)- obtained from Dr. Frank Lee of Stanford University with the permission of Dr. L. Chasin) with a vector expressing preproinsulin to obtain clones with reduced insulin requirements. These cells are also DHFR minus and clones can be selected for the presence of DHFR cDNA vector sequences by growth on medium devoid of nucleoside supplements (glycine, hypoxanthine, and thymidine). This selection system for stably expressing CHO cell lines is commonly used.

b. Transfection method (electroporation)

TPO_{332} and TPO_{153} expressing cell lines were generated by transfecting DP12 cells via electroporation (see e.g. Andreason, G.L. *J. Tiss. Cult. Meth., 15*, 56 (1993) with linearized pSVI5.ID.LL.MLORF or pSVI5.ID.LL.MLEPO-D plasmids respectively. Three (3) restriction enzyme reaction mixtures were set up for each plasmid cutting; 10 micrograms, 25 micrograms and 50 micrograms of the vector with the enzyme NOTI by standard molecular biology methods. This restriction site is found only once in the vector in the linearization region 3' and outside the TPO ligand transcription units. The 100 microliter reactions were set up for overnight incubation at 37 degrees. The next day the mixes were phenol-chloroform-isoamyl alcohol (50:49:1) extracted one time and ethanol precipitated on dry ice for approximately one hour. The precipitate was then collected by a 15 minute microcentrifugation and dried. The linearized DNA was resuspended into 50 microliters of Ham's DMEM-F12 1:1 medium supplemented with standard antibiotics and 2mM glutamine.

Suspension growing DP12 cells were collected, washed one time in the medium described for resuspending the DNA and finally resuspended in the same medium at a concentration of 10^7 cells per 750 microliters. Aliquots of cells (750 microliters) and each linearized DNA mix were incubated together at room temperature for one hour and then transferred to a BRL electroporation chamber. Each reaction mix was then electroporated in a standard BRL electroporation apparatus at 350 volts set at 33O micro F and low capacitance. After electroporation, the cells were allowed to sit in the apparatus for 5 minutes and then on ice for an additional 10 minute incubation period. The electroporated cells were transferred to 60mm cell culture dishes containing 5 ml of standard, complete growth medium for CHO cells (High glucose DMEM-F12 50:50 without glycine supplemented with

1X GHT, 2mM glutamine, and 5% fetal calf serum) and grown overnight in a 5% CO_2 cell culture incubator.

c. Selection and screening method

The next day, cells were trypsinized off the plates by standard methods and transferred to 150mm tissue culture dishes containing DHFR selective medium (Ham's DMEM-F12, 1:1medium described above supplemented with either 2% or 5% dialyzed fetal calf serum but devoid of glycine, hypoxanthine and thymidine this is the standard DHFR selection medium we use). Cells from each 60mm dish were subsequently replated into 5/150 mm dishes. Cells were then incubated for 10 to 15 days(with one medium change) at 37 degrees/15% CO_2 until clones began to appear and reached sizes amenable to transfer to 96 well dishes. Over a period of 4-5 days, cell lines were transferred to 96 well dishes using sterile yellow tips on a pipettman set at 50 ml. The cells were allowed to grow to confluency (usually 3-5 days) and then the trays were trypsinized and 2 copies of the original tray were reproduced. Two of these copies were short term stored in the freezer with cells in each well diluted into 50 microliter pf 10% FCS in DMSO. 5 day conditioned serum free medium samples were assayed from confluent wells in the third tray for TPO expression via the Ba/F cell based activity assay. The highest expressing clones based on this assay were revived from storage and scaled up to 2 confluent 150mm T-flasks for transfer to the cell culture group for suspension adaptation, re-assay and banking.

d. Amplification Protocol

Several of the highest titer cell lines from the selection described above were subsequently put through a standard methotrexate amplification regime to generate higher titer clones. CHO cell clones are expanded and plated in 10cm dishes at 4 concentrations of methotrexate (i.e 50 nM, 100 nM, 200 nM and 400 nM) at two or three cell numbers (10^5, $5x10^5$, and 10^6 cells per dish). These cultures are then incubated at 37 degree/5% CO_2 until clones are established and amenable to transfer to 96 well dishes for further assay. Several high titer clones from this selection were again subjected to greater concentrations of methotrexate (i.e. 600 nM, 800 nM, 1000 nM and 1200 nM) and as before resistant clones are allowed to establish and then transferred to 96 well dishes and assayed.

4. Culturing Stable CHO Cell Lines Expressing Recombinant Human TPO_{332} and TPO_{153}

Banked cells are thawed and the cell population is expanded by standard cell growth methods in either serum free or serum containing medium. After expansion to sufficient cell density, cells are washed to remove spent cell culture media. Cells are then cultured by any standard method including; batch, fed-batch or continuous culture at 25-40°C, neutral pH, with a dissolved O_2 content of at least 5% until the constitutively secreted TPO is accumulated. Cell culture fluid is then separated from the cells by mechanical means such as centrifugation.

5. Purification of Recombinant Human TPO from CHO Culture Fluids

Harvested cell culture fluid (HCCF) is directly applied to a BLUE-SEPHAROSE 6 FAST FLOW column (Phamacia) equilibrated in 0.01 M Na phosphate pH 7.4, 0.15M NaCl at a ratio of approximately 100 L of HCCF per liter of resin and at a linear flow rate of approximately 300 ml/hr/cm^2. The column is then washed with 3 to 5 column volumes of equilibration buffer followed by 3 to 5 column volumes of 0.01 M Na phosphate pH 7.4, 2.0 M urea. The TPO is then eluted with 3 to 5 column volumes of 0.01 M Na phosphate pH 7.4, 2.0M urea, 1.0M NaCl. The BLUE-SEPHAROSE pool containing TPO is then applied to a wheat germ lectin SEPHAROSE 6 MB column (Pharmacia) equilibrated in 0.01 M Na phosphate pH 7.4, 2.0M urea, and 1.0M NaCl at a ratio of from 8 to 16 ml of BLUE-SEPHAROSE pool per ml of resin at flow rate of approximately 50 ml/hr/cm^2. The column is then washed with 2 to 3 column volumes of equilibration buffer. The TPO is then eluted with 2 to 5 column volumes of 0.01 M Na phosphate pH 7.4, 2.0M urea, 0.5 M N-acetyl-D-glucosamine.

The wheat germ lectin pool is then adjusted to a final concentration of 0.04%$C_{12}E_8$ and 0.1% trifluroacetic acid (TFA). The resulting pool is applied to a C4 reverse phase column (Vydac 214TP1022) equilibrated in 0.1% TFA, 0.04% $C_{12}E_8$ at a load of approximately 0.2 to 0.5 mg protein per ml of resin at a flow rate of 157 ml/hr/cm^2.

The protein is eluted in a two phase linear gradient of acetonitrile containing 0.1% TFA, 0.04% $C_{12}E_8$. The first phase is composed of a linear gradient from O to 30% acetonitrile in 15 minutes, the second phase is composed of a linear gradient from 30 to 60% acetonitrile in 60 minutes. The TPO elutes at approximately 50% acetonitrile. A pool is made on the basis of SDS-PAGE.

The C4 pool is then diluted with 2 volumes of 0.01 M Na phosphate pH 7.4, 0.15 M NaCl and diafiltered versus approximately 6 volumes of 0.01 M Na phosphate pH 7.4, 0.15 M NaCl on an AMICOM YM or like ultrafiltration membrane having a 10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be then directly processed or further concentrated by ultrafiltration. The diafiltrate/concentrate is adjusted to a final concentration of 0.01% TWEEN-80.

All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the calculated column volume is then applied to a SEPHACRYL S-300 HR column (Pharmacia) equilibrated in 0.01 M Na phosphate pH 7.4, 0.15M NaCl, 0.01% TWEEN-80 and chromatographed at a flow rate of approximately 17 ml/hr/cm^2. The TPO containing fractions which are free of aggregate and proteolytic degradation products are pooled on the basis of SDS-PAGE. The resulting pool is filtered on a 0.22 micron filter, MILLEX-GV or like, and stored at 2-8°C.

EXAMPLE 3 - Transformation and Induction of TPO Protein Synthesis In E. coli

1. Construction of E. coli TPO expression vectors

The plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 are all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for high level translation initiation and rapid purification. The

plasmids pMP210-1, -T8, -21, -22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in *E. coli* upon induction of the tryptophan promoter (Yansura, D. G. et. al. *Methods in Enzymology* (Goeddel, D. V., Ed.) *185*:54-60, Academic Press, San Diego (1990)). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids.

(a) Plasmid pMP1

The plasmid pMP1 is a secretion vector for the first 155 amino acids of TPO, and was constructed by ligating together 5 fragments of DNA. The first of these was the vector pPho21 in which the small MluI-BamHI fragment had been removed. pPho21 is a derivative of phGH1 (Chang, C. N. *et. al., Gene 55*:189-196 (1987) in which the human growth hormone gene has been replaced with the E. coli phoA gene, and a MluI restriction site has been engineered into the coding sequence for the STII signal sequence at amino acids 20-21.

The next two fragments, a 258 base pair HinfI-PstI piece of DNA from pRK5-hmpI encoding TPO amino acids 19-103, and the following synthetic DNA encoding amino acids 1-18

5'-CGCGTATGCCAGCCCGGCTCCTCCTGCTTGTGACCTCCGAGTCCTCAGTAAACTGCTTCG TG (SEQ ID NO: 7)

ATACGGTCGGGCCGAGGAGGACGAACACTGGAGGCTCAGGAGTCATTTGACGAAGCACT GA-5' (SEQ ID NO:8)

were preligated with T4-DNA ligase, and second cut with PstI. The fourth was a 152 base pair PstI-HaeIII fragment from pRK5hmpII encoding amino acids 104-155 of TPO. The last was a 412 base pair StuI-BamHI fragment from pdh108 containing the lambda to transcriptional terminator as previously described (Scholtissek, S. et. al., *NAR 15*:3185 (1987)).

(b) Plasmid pMP21

The plasmid pMP21 is designed to express the first 155 amino acids of TPO with the aid of a 13 amino acid leader comprising part of the STII signal sequence. It was constructed by ligating together three (3) DNA fragments, the first of these being the vector pVEG31 in which the small XbaI-SphI fragment had been removed. The vector pVEG31 is a derivative of pHGH207-1 (de Boer, H. A. et. al., in *Promoter Structure and Function* (Rodriguez, R. L. and Chamberlain, M. J., Ed), 462, Praeger, New York (1982)) in which the human growth hormone gene has been replaced by the gene for vascular endothelial growth factor (this identical vector fragment can be obtained from this latter plasmid).

The second part in the ligation was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTTCTTCTTAA (SEQ ID NO:9)

TTAATACTTTTTCTTATAGCGTAAAGAAGAATTGCGC-5' (SEQ ID NO:10)

The last piece was a 1072 base pair MluI-SphI fragment from pMP1 encoding 155 amino acids of TPO.

(c) Plasmid pMP151

The plasmid pMP151 is designed to express the first 155 amino acids of TPO downstream of a leader comprising 7 amino acids of the STII signal sequence, 8 histidines, and a factor Xa cleavage site. pMP151 was constructed by ligating together three DNA fragments, the first of these being the previously described vector pVEG31 from which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTTCATCACCATCACCATCACCATCACATCGAA GGTCGTAGCC (SEQ ID NO:11)

TTAATACTTTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTCCAGCAT-5' (SEQ ID NO:12)

The last was a 1064 base pair BgLI-SphI fragment from pMP11 encoding 154 amino acids of TPO. The plasmid pMP11 is identical to pMP1 with the exception of a few codon changes in the STII signal sequence(this fragment can be obtained from pMP1).

(d) Plasmid pMP202

The plasmid pMP202 is very similar to the expression vector pMP151 with the exception that the factor Xa cleavage site in the leader has been replaced with a thrombin cleavage site. As shown in Fig. 36, pMP202 was constructed by ligating together three DNA fragments. The first of these was the previously described pVEG31 in which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTTCATCACCATCACCATCACCATCACATCGAA CCACGTAGCC (SEQ ID NO:13)

TTAATACTTTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTTGGTGCA T-5' (SEQ ID NO:14)

The last piece was a 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

(e) Plasmid pMP172

The plasmid pMP172 is a secretion vector for the first 153 amino acids of TPO, and is an intermediate for the construction of pMP210. pMP172 was prepared by ligating together three DNA fragments, the first of which was the vector pLS321amB in which the small EcoRI-HindI section had been removed. The second was a 946 base pair EcoRI-HgaI fragment from the previously described plasmid pMP11. The last piece was a synthetic DNA duplex with the following sequence:

5'-TCCACCCTCTGCGTCAGGT (SEQ ID NO:15)

GGAGACGCAGTCCATCGA-5' (SEQ ID NO:16)

(f) Plasmid pMP210

The plasmid pMP210 is designed to express the first 153 amino acids of TPO after a translational initiation methionine. This plasmid was actually made as a bank of plasmids in which the first 6 codons of TPO were randomized in the third position of each codon, and was constructed by the ligation of three DNA fragments. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex shown below treated first with DNA polymerase (Klenow) followed by digestion with XbaI and HinI, and encoding the initiation methionine and the randomized first 6 codons of TPO.

5'-GCAGCAGTTCTAGAATTATGTCNCCNGCNCCNCCNGCNTGTGACCTCCGAACACTGGAG GCTGTTCTCAGTAAA (SEQ ID NO:17)

CAAGAGTCATTTGACGAAGCACTGAGGGTACAGGAAG-5' (SEQ ID NO:18)

The third was a 890 base pair HinfI-SphI fragment from pMP172 encoding amino acids 19-153 of TPO.

The plasmid pMP210 bank of approximately 3700 clones was retransformed onto high tetracycline (50 microgram/ml) LB plates to select out high translational initiation clones (Yansura, D.G. et al., Methods: *A Companion to Methods in Enzymology 4*:151-158 (1992)). Of the 8 colonies which came up on high tetracycline plates, five of the best in terms of TPO expression were subject to DNA sequencing.

(g) Plasmid pMP41

The plasmid pMP41 is designed to express the first 155 amino acids of TPO fused to a leader consisting of 7 amino acids of the STII signal sequence following by a factor Xa cleavage site. The plasmid was constructed by ligating together three pieces of DNA, the first of which was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following synthetic DNA duplex:

5'-CTAGAATTATGAAAAAGAATATCGCATTTATCGAAGGTCGTAGCC (SEQ ID NO:19)

TTAATACTTTTTCTTATAGCGTAAATAGCTTCCAGCAT-5N (SEQ ID NO:20)

The last piece of the ligation was the 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

(h) Plasmid pMP57

The plasmid pMP57 expresses the first 155 amino acids of TPO downstream of a leader consisting of 9 amino acids of the StII signal sequence and the dibasic site Lys-Arg. This dibasic site provides for a means of removing the leader with the protease ArgC. This plasmid was constructed by ligating together three DNA pieces. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following synthetic DNA duplex:

5'-NCTAGAATTATGAAAAAGAATATCGCATTTCTTCTTAAACGTAGCC (SEQ ID NO:21)

TTAATACTTTTTCTTATAGCGTAAAGAAGAATTTGCAT-5N (SEQ ID NO:22)

The last part of the ligation was the 1064 base pair Bgil-Sphl fragment from the previously described plasmid pMP11.

(i) Plasmid pMP251

The plasmid pMP251 is a derivative of pMP210-1 in which two additional amino acids of TPO are included on the carboxy terminal end. This plasmid was constructed by ligating together two pieces of DNA, the first of these being the previously described pMP21 in which the small Xbal-Apal fragment had been removed. The second part of the ligation was a 316 base pair Xbal-Apal fragment from pMP210-1.

2. Transformation and Induction of E. co/i with TPO expression vectors

The above TPO expression plasmids were used to transform the E. coli strain 44C6 (w3110 tonA Δ rpoHts lon Δ cip Δ galE) using the $CaCl_2$ heat shock method (Mandel, M. *et al., J. Mol. Biol., 53*:159-162, (1970)). The transformed cells were grown first at 37°C in LB media containing 50 pg/ml carbenicillin until the optical density (600nm) of the culture reached approximately 2-3. The LB culture was then diluted 20x into M9 media containing 0.49% casamino acids (w/v) and 50 pg/ml carbenicillin. After growth with aeration at 30°C for 1 hour, indole-3-acrylic acid was added to a final concentration of 50 1lg/ml. The culture was then allowed to continue growing at 30°C with aeration for another 15 hours at which time the cells were harvested by centrifugation.

EXAMPLE 4 - Production of Biologically Active TPO (Met^{-1} 1-153) in *E. coli.*

The procedures given below for production of biologically active, refolded TPO (Met^{1}-153) can be applied in analogy for the recovery of other TPO variants including N and C terminal extended forms.

1. Recovery of non-soluble TPO (Met^{-1} 1-153)

E. coli cells expressing TPO (Met^{-1} 1-153) encoded by the plasmid pMP210-1 are fermented as described above. Typically, about 100g of cells are resuspended in 1 (10 volumes) of cell disruption buffer (10 mM Tris, 5 mM EDTA, pH 8) with a Polytron homogenizer and the cells centrifuged at 5000 x g for 30 minutes. The washed cell pellet is again resuspended in 1 L cell disruption buffer with the Polytron homogenizer and the cell suspension is passed through an LH CELL DISRUPTER (LH Inceltech, Inc.) or through a MICROFLUIDIZER (Microfluidics International) according to the manufactures' instructions. The suspension is centrifuged at 5,000 x g for 30 min. and resuspended and centrifuged a second time to make a washed refractile body pellet. The washed pellet is used immediately or stored frozen at -70°C

2. Solubilization and purification of monomeric TPO Met^{-1} 1-153)

solubilization of the TPO protein. High concentrations of urea (6-8M) are also useful but generally result in lower yields compared to guanidine. After solubilization, the solution is centrifuged at 30,000 x g for 30 min. to produce a clear supernatant containing denatured, monomeric TPO protein. The supernatant is then chromatographed on a SUPERDEX 200 gel filtration column (Pharmacia, 2.6 x 60 cm) at a flow rate of 2 ml/min. and the protein eluted with 20 mM Na phosphate, pH 6.0, with 10 mM DTT fractions containing monomeric, denatured TPO protein eluting between 160 and 200 ml are pooled. The TPO protein is further purified on a semi-preparative C4 reversed phase column (2 x 20 cm VYDAC). The sample is applied at 5 ml/min. to a column equilibrated in 0.1% TFA(trifluoroacetic acid) with 30% acetonitrile. The protein is eluted with a linear gradient of acetonitrile (30-60% in 60 min.). The purified reduced protein elutes at approximately 50% acetonitrile. This material is used for refolding to obtain biologically active TPO variant.

3. Generation of biologically active TPO (Met^{-1} 1-153)

Approximately 20 mg of monomeric, reduced and denatured TPO protein in 40 ml 0.1% TFA/50% acetonitrile is diluted into 360 ml of refolding buffer containing optimally the following reagents:

50 mM Tris
0.3 M NaCl
5 mM EDTA
2% CHAPS detergent
25% glycerol
5 mM oxidized glutathione
1 mM reduced glutathione
pH adjusted to 8.3

After mixing, the refolding buffer is gently stirred at 4°C for 12-48 hr to effect maximal refolding yields of the correct disulfide-bonded form of TPO (see below). The solution is then acidified with TFA to a final concentration of 0.2%, filtered through a 0.45 or 0.22 micron filter, and 110 volume of acetonitrile added. This solution is then pumped directly onto a C4 reversed phase column and the purified, refolded TPO (Met^{-1} 1-153) eluted with the same gradient program as above. Refolded, biologically active TPO is eluted at approximately 45% acetonitrile under these conditions. Improper disulfide-bonded versions of TPO are eluted earlier. The final purified TPO (Met^{-1} 1-153) is greater than 95% pure as assessed by SDS gels and analytical C4 reversed phase chromatography. For animal studies, the C4 purified material was dialyzed into physiologically compatible buffers. Isotonic buffers (10 mM Na acetate, pH 5.5, 10 mM Na succinate, pH 5.5 or 10 mM Na phosphate, pH 7.4) containing 150 mM NaCl and 0.01% TWEEN-80 were utilized.

Because of the high potency of TPO in the Ba/F3 assay (half maximal stimulation is achieved at approximately 3 pgml), it is possible to obtain biologically active material utilizing many different

buffer, detergent and redox conditions. However, under most conditions only a small amount of properly folded material (<10%) is obtained. For commercial manufacturing processes, it is desirable to have refolding yields at least 10%, more preferably 30-50% and most preferably >50%. Many different detergents (TRITON X-100, dodecyl-beta-maltoside, CHAPS, CHAPSO, SDS, SARKOSYL, TWEEN-20 and TWEEN-80, ZWITTERGENT 3-14 and others) were assessed for efficiency to support high refolding yields. Of these detergents, only the CHAPS family (CHAPS and CHAPSO) were found to be generally useful in the refolding reaction to limit protein aggregation and improper disulfide formation. Levels of CHAPS greater than 1% were most useful. Sodium chloride was required for best yields, with the optimal levels between 0.1 M and 0.5M. The presence of EDTA (1-5 mM) limited the amount of metal-catalyzed oxidation (and aggregation) which was observed with some preparations. Glycerol concentrations of greater than 15% produced the optimal refolding conditions. For maximum yields, it was essential to have both oxidized and reduced glutathione or oxidized and reduced cysteine as the redox reagent pair. Generally higher yields were observed when the mole ratio of oxidized reagent is equal to or in excess over the reduced reagent member of the redox pair pH values between 7.5 and about 9 were optimal for refolding of these TPO variants. Organic solvents (e.g. ethanol, acetonitrile, methanol) were tolerated at concentrations of 10-15% or lower. Higher levels of organic solvents increased the amount of improperly folded forms. Tris and phosphate buffers were generally useful. Incubation at 4°C also produced higher levels of properly folded TPO.

Refolding yields of 40-60% (based on the amount of reduced and denatured TPO used in the refolding reaction) are typical for preparations of TPO that have been purified through the first C4 step. Active material can be obtained when less pure preparations (e.g. directly after the Superdex 200 column or after the initial refractile body extraction) although the yields are less due to extensive precipitation and interference of non-TPO proteins during the TPO refolding process.

Since TPO (Met^{-1} 1-153) contains 4 cysteine residues, it is possible to generate three different disulfide versions of this protein:

version 1: disulfides between cysteine residues 1-4 and 2-3
version 2: disulfides between cysteine residues 1-2 and 3-4
version 3: disulfides between cysteine residues 1-3 and 2-4.

During the initial exploration in determining refolding conditions, several different peaks containing the TPO protein were separated by C4 reversed phase chromatography. Only one of these peaks had significant biological activity as determined using the Ba/F3 assay. Subsequently, the refolding conditions were optimized to yield preferentially that version. Under these conditions, the misfolded versions are less than 10-20% of the total monomer TPO obtained.

The disulfide pattern for the biologically active TPO has been determined to be 1-4 and 2-3 by mass spectrometry and protein sequencing (i.e. version 1). Aliquots of the various C4-resolved peaks

(5-10 nmoles) were digested with trypsin (1:25 mole ratio of trypsin to protein). The digestion mixture was analyzed by matrix assisted laser desorption mass spectrometry before and after reduction with DTT. After reduction, masses corresponding to most of the larger tryptic peptides of TPO were detected. In the un-reduced samples, some of these masses were missing and new masses were observed. The mass of the new peaks corresponded basically to the sum of the individual tryptic peptides involved in the disulfide pair. Thus it was possible to unequivocally assign the disulfide pattern of the refolded, recombinant, biologically active TPO to be 1-4 and 2-3. This is consistent with the known disulfide pattern of the related molecule erythropoietin.

D. Biological activity of recombinant, refolded TPO (met 1-153)

Refolded and purified TPO (Met^{-1} 1-153) has activity in both *in vitro* and *in vivo* assays. In the Ba/F3 assay, half-maximal stimulation of thymidine incorporation into the Ba/F3 cells was achieved at 3.3 pg /ml (0.3 pM). In the *mpl* receptor-based ELISA, half-maximal activity occurred at 1.9 ng/ml (120 pM). In normal and myelosuppressed animals produced by near-lethal X-radiation, TPO (Met^{-1} 1-153) was highly potent (activity was seen at doses as low as 30 ng/mouse) to stimulate the production of new platelets.

EXAMPLE 5 - Myelosuppressed (Carboplatin/Irradiation) Mouse Data

Animals

All animal studies were approved by the Institutional Care and Use Committee of Genentech Inc. Prior to the start of the experiment all animals were ear-tagged for identification and a base-line complete blood count (CBC) obtained. Groups of 10 female C57BL/6 mice were irradiated with 5.0 Gy of gamma irradiation from a ^{137}Cs source. Within 6 hours, the animals were given 1.2 mg carboplatin as a 200 microliter intraperitoneal injection.

The following are the protocols and results using recombinant murine thrombopoietin (rmTPO) in a standard mouse model. It will be understood that one skilled in the art considers this model to correlate with treatment in human beings. Human thrombopoietin has been tested in the same mouse model and was found to show relevant activity, albeit at a lesser level because of the species specificity. Therefore, the following protocol was chosen using the proper murine TPO counterpart for that species so that relevant effect could be demonstrated. Again, use of human TPO in the mouse protocol would provide similar results differing only in degree.

Procurement of Blood Samples

Prior to the experiment and at time points throughout the study, 40 microliter of blood was taken from the orbital sinus and immediately diluted into 10 mL of diluent to prevent clotting. The complete blood count (CBC) from each blood sample was measured on a SERRANO Baker system

9018 blood analyzer within 60 min of collection. Only half of the animals in each dose group were bled on a given day; thus, each animal was bled on alternate time points.

Treatment Regimens

Experiment 1: In order to determine the response to recombinant murine thrombopoietin (rmTPO335aa) in animals rendered thrombocytopenic, groups of animals were treated for 1, 2, 4, or 8 consecutive days with 0.1 microgram/day (5 microgram/kg/day approx.). Treatment with rmTPO (335aa) was started 24 hours after the initiation of the model and was given as a daily 100 microliter subcutaneous injection.

Experiment 2: In order to determine the nature of the dose-response relationship for rmTPO(335) in this model, animals were given a single injection of rmTPO (335) 24 hours after the initiation of the model. Groups of animals received one of 0.01, 0.03, 0.1 or 0.3 microgram of rmTPO (335) as a single 100 microliter subcutaneous injection. In order to compare two routes of administration, a contemporaneous experiment used 4 groups of animals receiving identical doses of rmTPO (335) but via an intravenous route (lateral tail vein).

Experiment 3: This series of experiments was done to compare the efficacy of various pegylated truncated rmTPO molecules (rmTPO(153) coupled to polyethylene glycol (PEG).

i. In this experiment thrombocytopenic animals were injected (0.1 microgram subcutaneous) with one of the following pegylated rmTPO(153) molecules: no PEG, one 20K PEG or one 40K PEG.

ii. In the final experiment there was compared the effects of administering a single 40K PEG rmTPO(153) molecule by giving 0.1microgram either subcutaneously or intravenously to animals rendered thrombocytopenic. rmTPO(335) (0.1 microgram) was used as a positive control.

Results

The combination of sublethal irradiation and carboplatin resulted in a reproducible response giving consistent thrombocytopenia in 100% of the animals. The nadir for the thrombocytopenia occurred at day 10 with a gradual recovery of platelet numbers by day 21 to day 28. Accompanying this thrombocytopenia was a pronounced anemia with the nadir occurring slightly later on day 14 to 17 and recovery to control red blood cell counts by day 28. White blood cell counts were also depleted during the course of the experiment.

Experiment 1: A single dose of 0.1 microgram rmTPO(335) given 24 hours after the initiation of the model accelerated the recovery of platelet numbers in this murine model. This single administration of rmTPO(335) elevated the nadir of the response from $196x10^3 \pm 33x10^3$/microliter on day 10 to $434x10^3 \pm 7x10^3$/microliter on day 7. The initial rate of decline in the platelet numbers remained unchanged but the recovery phase was much more rapid with platelet numbers returning to normal by

day 14 as opposed to day 21 in the control group. Some further improvement in the rate of recovery was seen by giving 0.1 microgram/day on day 1 and day 2 but this was marginal. No further improvement could be seen by giving rmTPO(335) for 4 or 8 consecutive days (Fig. 1a). In addition to the accelerated recovery in platelet numbers, the anemia which develops in these animals was also attenuated by a single dose of rmTPO(335) given on day 1. As with the platelet counts, no further advantage could be gained by giving rmTPO(335) more than once (Fig. 1b). rmTPO(335) had no effect on the leukocytopenia that accompanies the falls in platelet and red blood cell counts. (Fig. 1c).

Experiment 2: The response to single subcutaneous doses of rmTPO(335) given 24 hours after the initiation of the model was dose dependent. The lowest dose tested (0.01 microgram) had no effect on the platelet recovery compared to controls. However, the response is almost maximal when 0.03 microgram was given (Fig. 2a). This extremely steep dose response curve is better appreciated when the platelet numbers on day 14 are plotted on a log-linear plot (Fig. 3a). A similar steep dose response is seen for erythrocyte repopulation in this model (Fig. 3b). Intravenous administration of rmTPO(335) gave a similar dose dependent response. However, the lowest dose tested (0.01 microgram) was effective when given IV, (Fig. 4a) suggesting that the dose response curve is shifted to the left. This increase in potency is small since the shift is less than half an order of magnitude (Fig. 3a). What is more important is that both routes of administration have the comparable maxima (Fig. 3a). The subcutaneous and intravenous route of administration also augmented the recovery from the anemia in a dose-dependent fashion (Figs. 2a, 3b, 4b). However, neither the subcutaneous nor the intravenous route of administration had an effect on the leukocytopenia over the dose range tested (Figs. 2c,4c).

Experiment 3:

A. Pegylation of the rmTPO(153) with either a single 20K PEG or a single 40K PEG had a greater effect on the platelet recovery than the un-pegylated molecule. Unlike the full-length molecule, neither of the pegylated rmTPO(153) molecules affected the nadir of the thrombocytopenia but greatly accelerated the recovery phase of the model when given as a single 0.1 microgram SC dose 24 hours after initiation of the model (Fig 5a). This is very evident on day 14 when the platelet counts are $80x10^3 \pm 15x10^3$/microliter, $268x10^3 \pm 67x10^3$/microliter, $697x10^3 \pm 297x10^3$/microliter, and $878x10^3 \pm 31x10^3$/microliter for controls, rmTPO(153) no PEG, rmTPO(153) + 20K PEG and rmTPO(153) + 40K PEG respectively (Fig. 5a). The same profile was also evident on the erythrocyte response (Fig. 5b). None of these rmTPO(153)-based molecules had any effect on the leukocytopenia in this model. (Fig. 5c).

B. rmTPO(153) + 40K PEG (0.1 microgram) gave a consistent response when administered as either a single intravenous or subcutaneous injection. In this experiment, the subcutaneous route slightly altered the nadir on day 10 and returned platelets to control levels by day 14 as compared to

day 28 in the control group (Fig. 6a). In the animals given the drug intravenously, there was a similar effect on the nadir and rate of recovery (Fig. 7a). The response to this 40K pegylated truncated rmTPO(153) molecule is almost identical to the response to the rmTPO(335) on both platelet and erythrocyte recovery when given either subcutaneously (Fig. 6b) or intravenously (Fig. 7b). As with all of the other experiments rmTPO(153) + 40K PEG given either subcutaneously or intravenously had no effect on the circulating levels of white blood cells (Figs. 6c, 7c). In parallel experiments, the use of 10K-pegylated versions of this molecule did not modify the response to rmTPO(153) on either platelet or erythrocyte repopulation.

EXAMPLE 6

The following are protocols and results using single-dose therapy with recombinant human thrombopoietin ($rhTPO_{332}$) in human patients receiving cytotoxic chemotherapy:
Single-dose therapy with recombinant human thrombopoietin (rhTPO) in patients receiving cytotoxic chemotherapy.

Preclinical models of intensive chemoradiotherapy demonstrated that a single dose of rhTPO raises the platelet nadir and shortens the period of severe thrombocytopenia. Interim results of two Phase I studies in which single doses of rhTPO were administered to cancer patients receiving chemotherapy are presented.

Patients and Methods:

Both studies began with 21-day, pre-chemotherapy periods (cycle 0) for assessment of rhTPO safety and platelet response after single IV bolus injections of 0.3, 0.6, or 1.2 meg/kg (3 patients per group in each study). Patients then received the same dose of rhTPO after chemotherapy in selected subsequent cycles. The first study population consisted of patients with advanced malignancies who received rhTPO the day following salvage thiotepa chemotherapy (65 mg/m^2 q28d) in each of two consecutive chemotherapy cycles. The second study included chemotherapy naive patients with sarcoma undergoing induction treatment with AI chemotherapy (doxorubicin 90 mg/m^2, 10 g/m^2 q2ld. Following cycle 0, patients in this study were monitored during the first chemotherapy cycle and received a single rhTPO injection the day following completion of chemotherapy (d5) during the second and subsequent cycles.

Results:

14 patients have been treated to date. rhTPO was well tolerated with no reported serious adverse events attributed to study drug. Antibodies to rhTPO have not been observed. In cycle 0 the lowest (0.3 mcg/kg) dose was weakly active, with increased activity at higher doses as shown below.

RhTPO dose (mcg/kg)	Patients N	Mean Baseline Patients (1μl) (SD)	Median Maximum Platelet (1μl) (Range)	Median % Increase
0.3	7	339 (133)	510 (277-628)	40
0.6	5	235 (69)	486 (386-509)	103
1.2	2	203 (46)	523 (437, 608)	158

The maximum platelet count during cycle 0 occurred on median day 11 (range 7-14). No significant changes were found in WBC or HCT. FACS analysis of bone marrow showed increases in all CD34+ subsets in 2/2 patients following 0.6 mcg/kg. Increases in peripheral blood CD34+ cells were also seen in these patients, suggesting that TPO might have stem cell mobilizing activity. Dose calculation and post-chemotherapy treatment are ongoing.

Together these phase 1 studies suggest that single dose administration of rhTPO is safe and well tolerated. The 0.3, 0.6. and 1.2 mcg/kg. dose levels show increasing thrombopoietic activity. The ongoing treatment of patients at higher dose levels will test the hypotheses that a single dose of rhTPO is efficacious in ameliorating thrombocytopenia following intensive chemotherapy.

EXAMPLE 7A - A Phase I Study To Determine The Safety, Tolerance, Pharmacokinetics And Pharmacodynamics Of Recombinant Human Thrombopoietin (rhTPO) In Subjects With Sarcoma Receiving Adriamycin And Ifosfamide

Treatment Plan

This was a single-center, open-label, dose-escalation, study of single and multiple IV doses of rhTPO with major safety endpoints. rhTPO was administered to subjects with histologically diagnosed sarcoma to determine whether its administration helped to prevent, delay, ameliorate, or shorten the duration of the known thrombocytopenic effects of doxorubicin and ifosfamide.

At present, 71 subjects have been enrolled on to this study which addresses the safety and the activity, of various rhTPO dosing schedule in conjuction with G-CSF and GM-CSF. The study begins with a 21-day pre-chemotherapy cycle (cycle 0) to assess safety, activity, and pharmacokinetics in patients with cancer. The data indicates a dose-dependent increase in peripheral blood platelet counts and bone marrow megakaryocytes in response to either a single or multiple IV doses of rhTPO, with doses ranging from 0.3-3.6 mg/kg. Thus rhTPO dosing regimens in which all of the rhTPO was administered after the completion of chemotherapy were safe, but demonstrated only modest activity. One patient with a normal platelet count developed an uncomplicated deep venous thrombosis in the leg which resolved with conservative therapy, and neutralizing antibodies have not been observed. Subjects had either metastatic or unresectable sarcoma.

In patients who have received a single dose of rhTPO prior to the chemotherapy have experienced a benefit in terms of reducing the depth and duration of the platelet nadir when compared

to patients in other dosing arms or historical controls, and this effect has been exhibited throughout the six cycles of planned chemotherapy (of note, none of the historical control patients (n=18) received all six cycles, and only two received five cycles). The prechemotherapy dose has been well-tolerated. As dosing through chemotherapy is safe and potentially more efficacious, this rhTPO dosing regimen is being incorporated into the protocol.

Dose Levels

Five dose levels of rhTPO and six regimens (Arms A-F) were evaluated in this study. Dose levels and the number of subjects per dose level are as shown in the Table below.

Dose Levels, Doses and Number of Subjects

LEVEL Dose		DOSE		N
1A	0.3	µg/kg	X 1	3
2A	0.6	µg/kg	X 1	3
3A	1.2	µg/kg	X 1	3
4A	2.4	µg/kg	X 1	4
5A	3.6	µg/kg	X 1	3
1B	0.3	µg/kg	X 2	6
2B	0.6	µg/kg	X 2	3
3B	1.2	µg/kg	X 2	4
4B	2.4	µg/kg	X 2	3
5B	3.6	µg/kg	X 2	3
3C	1.2	µg/kg	QDX7	3
4C	2.4	µg/kg	QDX7	3
3D	1.2	µg/kg	pre/post	6
4D	2.4	µg/kg	pre/post	6
OBD*-Chemo	1.2	µg/kg	pre/post	6
OBD-TPO	1.2	µg/kg	pre/post	6
3E TPO +GM-CSF	1.2	µg/kg	pre/post	6
3Fa	1.2	µg/kg	d -1,1, 4 + G-CSF	6
3Fb	1.2	µg/kg	d -1,1, 4 + GM-CSF	6
				83

*OBD--Optimal Biologic Dose

Peak elevations in platelet count after single- or multiple-dose administration in preclinical studies have been observed within several days after initiation of dosing. Therefore, a peak platelet response would be expected within 7-14 days after initiation of dosing in this study. Current clinical experience has supported these findings.

Study Design

Subjects were assigned to one of the dose groups described in the Table above. Study schemata for Arms A-F are shown below. All subjects received doxorubicin and ifosfamide in Cycle 1 (Days 0, 1, 2, and 3) and in additional cycles.

The rhTPO dosing regimens for this study are as follows:

Arm A

Cycle 0: rhTPO on Day 0

Cycle 1: no rhTPO

Cycle 2+: rhTPO on Day 4

Arm B

Cycle 0: rhTPO on Days 0 and 3

Cycle 1: no rhTPO

Cycle 2+: rhTPO on Days 4 and 7

Arm C

Cycle 1: no rhTPO

Cycle 2+: daily rhTPO on Days 4 through 10, or until the post-nadir platelet count is $^{3}100{,}000/\mu L$

Arm D

Cycle 1: no rhTPO

Cycle 2+: rhTPO on Days Ð1, 4, and 7

Arm E

Cycle 1: no rhTPO

Cycle 2+: rhTPO as per Arm D regimen, but with GM-CSF replacing G-CSF

Cycle 0: This phase of the study evaluates safety, pharmacokinetics, and pharmacodynamics of single and multiple dosing. Subjects in Arm A received a single IV injection of rhTPO (Day 0). Subjects in Arm B received an IV injection of rhTPO on Days 0 and 3.

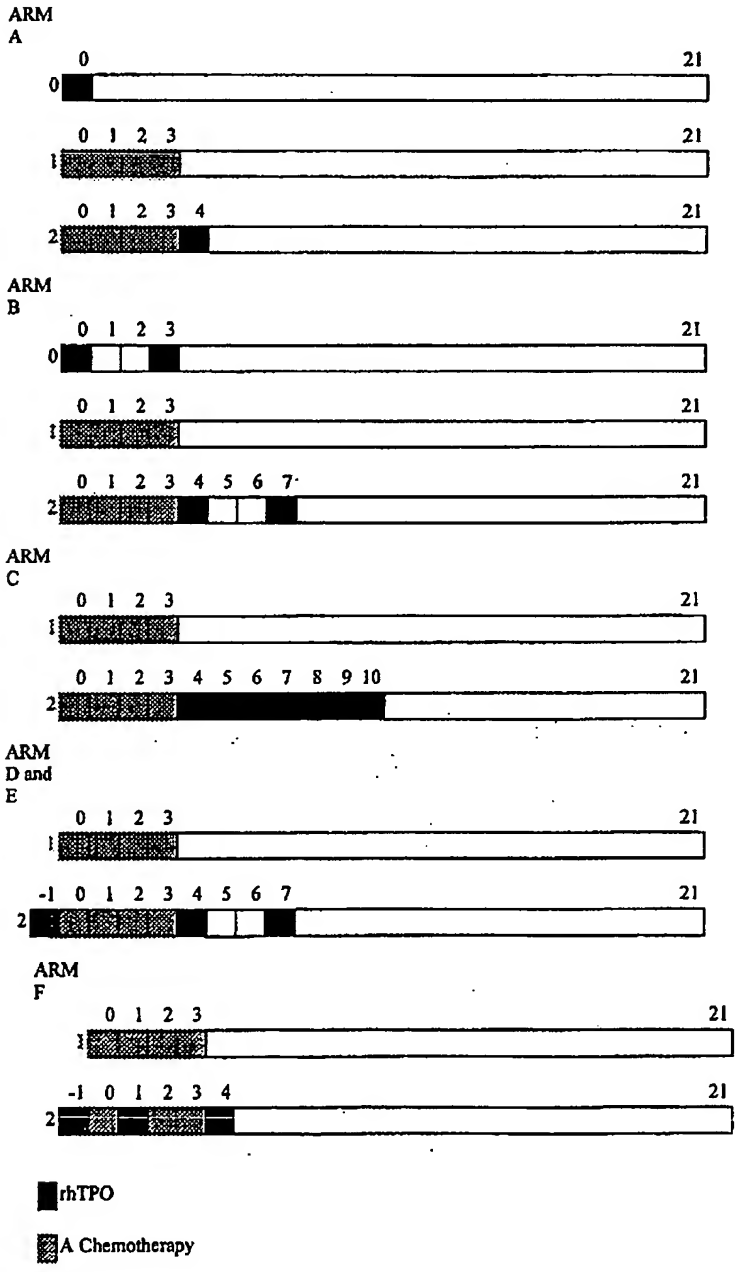
Cycle 1: Subjects received doxorubicin and ifosfamide. There was no rhTPO administered during Cycle 1. This provides a control cycle for comparison of each subject's response to subsequent cycles with rhTPO. This control cycle also clarifies chemotherapy-related adverse events in the absence of rhTPO dosing.

Cycle 2+: Subjects received doxorubicin and ifosfamide. Subjects in Arm A received a single IV injection of rhTPO (Day 4). Subjects in Arm B received IV injections of rhTPO on Days 4 and 7.

Subjects in Arm C received up to seven daily IV injections of rhTPO on Days 4-10, or until the post-nadir platelet count was greater than or equal to 100,000/μL. Subjects in Arm D received IV injections of rhTPO on Day -1 (1 day prior to chemotherapy) and on Days 4 and 7.

To explore a possible synergistic relationship of rhTPO with GM-CSF in humans while minimizing subject risk, GM-CSF replaced G-CSF in Arm E. GM-CSF was administered in Cycles 1 and 2, and in subsequent cycles where there is proven benefit. Subjects in Arm E received rhTPO according to the Arm D regimen.

Study Schema



Doxorubicin

Doxorubicin was obtained from commercial sources and stored and administered in accordance with the manufacturer's guidelines. Doxorubicin (a total of 90 mg/m^2) was given as a continuous infusion for the first 3 days of each chemotherapy cycle (30 mg/m^2 daily for 3 days, (Days 0-2).

Ifosfamide

Ifosfamide was obtained from commercial sources and stored and administered in accordance with the manufacturer's guidelines. Ifosfamide (a total of 10 g/m^2) was given as four separate daily infusions during the first 4 days of each chemotherapy cycle (2.5 g/m^2 over 3 hours daily for 4 days, (Days 0-3).

Mesna

Mesna was obtained from commercial sources and stored and administered in accordance with the manufacturer's guidelines. Mesna (500 mg/m^2 or 20% of the dose of ifosfamide) was administered IV over 3 hours with the initial dose of ifosfamide on Day 0 of each chemotherapy cycle. Mesna administration was maintained as a continuous IV infusion (1500 mg/m^2/day for a total of 6 g/m^2) until 24 hours after the final ifosfamide administration of each chemotherapy cycle (Days 0-4).

G-CSF

G-CSF was obtained from commercial sources and stored and administered in accordance with the manufacturer's guidelines. G-CSF (5 µg/kg) was administered daily beginning on Day 4 of Cycle 1 and any subsequent chemotherapy cycles. Subjects were instructed to self-administer G-CSF. All injections of G-CSF should be administered at ~8:00 p.m. Injections on the same day as rhTPO administration should be given ~12 hours after rhTPO administration to help define any injection-related phenomena. G-CSF administration continued on a daily basis until the absolute neutrophil count was >1500/µL for at least two consecutive measurements post-nadir.

GM-CSF

GM-CSF is obtained from commercial sources and stored and administered in accordance with the manufacture's guidelines. GM-CSF (250 mg/m^2) is administered subcutaneously daily beginning on Day 4 of Cycle 1 and any subsequent chemotherapy cycles. Subjects are instructed to self-administer GM-CSF. All injections of GM-CSF should be administered at ~8:00 p.m. Injections on the same day as rhTPO administrations should be given ~12 hours after rhTPO administration to

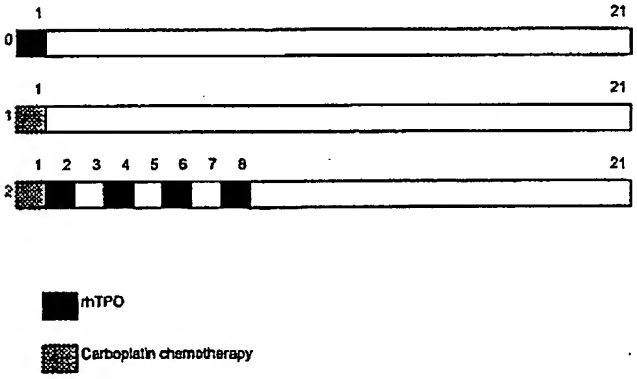
help define any injection-related phenomena. GM-CSF administration continued on a daily basis until the post-nadir absolute neutrophil count is >1500/μL for at least two consecutive measurements.

The results of this study are shown in Figures 14 - 19.

EXAMPLE 7B - A Phase I Trial Of Recombinant Human Thrombopoietin (rhTPO) Given Via Subcutaneous Injection To Subjects With Advanced Gynecologic Malignancy Receiving Carboplatin

This study addresses the safety of rhTPO administered subcutaneously and the activity of rhTPO dosing in an every other day schedule. The study begins with a 21-day pre-chemotherapy cycle (cycle 0) to assess safety, activity, and pharmacokinetics in patients with cancer. At present, 16 subjects have been enrolled on to this trial. The data indicates a dose-dependent increase in peripheral blood platelet counts in cycle 0, though the platelet responses, and the pharmacokjinetics, are blunted when compared to similar doses of IV rhTPO. Thus far, two antibodies to the truncated form of rhTPO (erythropoietin-like domain) have been observed; one was preformed, and neither was neutralizing in a bioassay, or clinically. Subjects had recurrent or advanced gynecologic neoplasms and were receiving carboplatin.

Schema



Dose Levels, Doses and Number of Subjects

DOSE LEVEL		DOSE		N
1A	0.6	μg/kg	QOD X4	3
2A	1.2	μg/kg	QOD X4	3
3A	2.4	μg/kg	QOD X4	3
4A	3.6	μg/kg	QOD X4	3
Control*	1.2	μg/kg	QOD X4	6
				18

*Control--patients receive rhTPO only in cycles after experiencing a platelet count ≤ 30/000/μl in the preceding cycle

EXAMPLE 8

Animals: 76 CD-1 female mice BW= 19.7-26.3 g

Aerosol Exposure:

Mice were exposed to rhTPO in a nose-only inhalation (CH Technologies) with simultaneous measurement of breathing pattern (body plethysmograph) for a total duration of 60 minutes. A PARI-IS2 nebulizer was used to aerosolize rhTPO at 22 psi and a flow rate of 5.1 LPM. Filter samples were taken to estimate the aerosol concentration for each exposure. A vehicle control group and 3 different concentrations (0.05, 0.5, and 5.0 mg/ml rhTPO) were nebulized. The dose groups are expressed as the estimated amount per kg that deposited in the mouse lungs for each group. Half of the animals were exposed only once (Single exposure) while the other half were exposed to rhTPO 3 times on days 0, 2 and 6. Anti-rhTPO antibodies were measured in only the highest dose group, but these antibodies were not neutralizing. The dose groups are shown in the table below.

Dose groups:

(rhTPO) Nebulized (mg/ml)	n	Estimated Lung Dose (mg/kg)
0	17	0
0.05	17	6.4
0.5	17	64
5.0	16	640

Data Endpoints:

Serum and blood were collected at -4, 3, 6, 8, 10, 14, 21, 30, and 43 days post aerosol inhalation for hematology (platelet counts) and measurement of serum anti-TPO antibodies.

Estimation of Deposited rhTPO Lung Dose:

Deposited dose (µg/kg)= Chamber concentration (µg/ml) x Minute volume (ml/min) x time of exposure (min) x Deposition fraction / BW (kg)

where: Chamber concentration = 0.000839, 0.00839, or 0.0839 µg/ml

Minute volume = 30 ml/min

Time of exposure = 60 min

Deposition fraction = 0.1

Body weight = .023 kg

Deposited dose = 6.4, 64, or 640 µg/kg (for 0.05, 0.5, or 5 mg/ml solutions). Platelet counts for mice exposed to a single inhalation (dose) of rhTPO are shown in Figure 20. Platelet counts for mice exposed to multiple inhalations (dose) of rhTPO are shown in Figure 21.

EXAMPLE 9

Animals: Female (C57BLxCBA)F1 (BCBA) mice approximately 12 weeks old were bred at the Experimental Animal Facility of the Erasmus University, Rotterdam, The Netherlands, and maintained under SPF conditions. Housing, experiments and all other conditions were approved by an ethical committee in accordance with legal regulations in The Netherlands.

Experimental setup: TBI was given at day 0 using a two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) at a dose rate between 0.92 and 0.94 Gy/min. Doses used were 6 Gy for the single dose irradiation and a total dose of 9 Gy was split in three doses of 3 Gy given with 24 hour intervals. For each data point groups of three mice were killed. All parameters were collected for individual mice.

Test Drug: Recombinant full length murine TPO produced by CHO cells (Genentech Inc., South San Francisco, CA was used throughout the experiments, diluted in PBS/0.01% TWEEN-20 and administered intraperitoneally in a volume of 0.5 ml.

TPO levels: Data for characterization plasma TPO pharmacokinetics were generated at Genentech, Inc. as previously described. In short, mice were injected i.p. with ^{125}I-rmTPO either with a single dose of 0.9 microgram/mouse (ca 45 microgram/kg) or with three doses of 0.3 microgram/mouse (ca 15 microgram/kg) separated by 24 hours. Citrated blood was collected immediately after dosing and at intervals thereafter (n=3 mice per timepoint), centrifugated at 2950 x g for 10 minutes, plasma harvested, and TCA- precipitable radioactivity determined. Pharmacokinetic parameters were estimated after converting TCA-precipitable cpm/mL and fitting the concentration versus time data to a two-compartment model with first order absorption using nonlinear least-squares regression analysis (WIN-NONLIN; Statistical Consultants, Lexington, KY). Area under the concentration time curves (AUC), maximum concentration (Cmax), terminal half-lives (t1/2), and clearance (mL/hr/kg) were calculated using coefficients and exponents obtained from the model fits.

Hematological examinations: After either-anesthesia the mice were bled by retro-orbital puncture and killed by cervical dislocation. Blood was collected in EDTA tubes. Complete blood cell counts were measured using a SYSMEX F-800 hematology analyzer (Toa Medical Electronics Co., LTD., Kobe, Japan). Differential white blood cell counts were performed after May-Grünwald-Giemsa staining.

Colony assays: Serum free methylcellulose cultures were used in this study. Appropriate numbers of bone marrow cells were suspended in Dulbecco's modified Eagle's medium (Dulbecco's MEM) obtained from GIBCO (Life Technologies LTD, Paisley, Scotland) supplemented with the amino acids L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid and L-proline (Sigma), vitamin B12, biotin, Na-pyruvate, glucose, $NaHCO_3$, and antibiotics (penicillin and streptomycin) at an osmolarity of 300 mOsm/l (∝-medium). Appropriate numbers of cells in ∝-medium containing 0.8% methylcellulose (Methocel A4M Premium Grade, Dow Chemical Co.,

Barendrecht, The Netherlands), 1% bovine serum albumin (BSA, fraction V, Sigma), $2x10^{-6}$ mol/l iron saturated human transferrin (Intergen Company N.Y., NY), 10^{-7} mol/l N_2SeO_3 (Merck), 10^{-4} mol/l β-mercapto-ethanol (Merck, linoleic acid (Merck), and Cholesterol (Sigma) at a final concentration between 7.5×10^{-6} mol/l and 1.5×10^{-5} mol/l for both, depending on the kind of progenitor cell colony cultured and 10^{-3} g/l nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyadenosine, thymidine and 2'-deoxyguanosine obtained from Sigma) were plated in 35 mm Falcon 1008 Petri dishes (Bercton Dickinson Labware) in 1 ml. aliquots.

Granulocyte/macrophage colony formation was stimulated by a saturating concentration of M-CSF purified from pregnant mouse uteri extract (PMUE) essentially as described before, supplemented with 100 ng/ml murine stem cell factor (SCF, a kind gift from Immunex Corporation, Seattle, WA) and 10 ng/ml murine IL-3 (R&D, Minneapolis, MN). GM-CFU colonies were counted after 7 days of culture.

BFU-E growth was stimulated by 100 ng/ml DCF and 4 U/ml murine erythropoietin (EPO, Behringwerke, Marburg, F.R.G.) purified from the serum of phenylhydrazine treated mice, titrated to an optimal concentration. Colonies were counted after 10 days of culture. The culture medium of the erythroid progenitors also contained hermine (bovine, type I, Sigma) at a concentration of $2x\ 10^{-4}$ mol/l.

Megakaryocyte progenitor cells (CFU-Meg) were cultured in 0.25% agar cultures. Colony formation was stimulated by 100 ng/ml SCF, 10ng/ml IL-3 and 10 ng/ml murine TPO (Genentech, Inc., San Francisco, CA). After 10 days colonies were dried, stained for acetylcholinesterase positive cells and counted. All cultures were grown in duplicate at 37 °C in a fully humidified atmosphere with 10% CO_2 in the air. Colony numbers represent the mean ± standard deviation of bone marrow samples of individual mice.

The spleen colony assay: This assay was performed as described by Till and McCulloch. Briefly, mice were injected with 5×10^4 BM cells or 5×10^5 spleen cells in HH one day after TBI. Thirteen days later, mice were sacrificed, and spleens were excised and fixed in Tellyesniczsky's solution (64% ethanol, 5% acetic acid and 2% formaldehyde) in H_2O.

Statistics: Standard deviations were calculated and are given in the text and the figures on the assumption of a normal distribution. The significance of a difference was calculated by one way analysis of variance followed by a non-paired Student's t test using STATVIEW, Abacus Concepts Inc., Berkeley, CA. All colony assays were done in duplicate for individual mice. The results of the colony assays are expressed as the means ± ISD per femur or spleen for at least three mice per group.

Experimental Design 1: BCBA F1 mice were subjected to 3 Gy total body irradiation at time 0. 0.3 microgram TPO/mouse was administered i.p. at various time points as indicated in Figure 8 or 30 microgram TPO/mouse i.p. at -2 h.

Figure 8 shows the thrombocyte level of 6 Gy irradiated mice at the time of the nadir in the placebo controls as a function of the time of administration of a single dose of TPO. Based on the kinetics observed, it appears that the peak levels of TPO reached shortly after TPO administration were the most relevant for efficacy. This was directly confirmed by administration of a very high dose of TPO 2 h before TBI (Fig. 9). The effect originated from multilineage cells, as shown in the peripheral blood by equivalent effects of TPO on red cells, white cells (mainly neutrophils) and platelets. See Table 1 below.

Table 1 - Major peripheral blood cell counts 10 days after 6 GY TBI and TPO administration

Time relative to TBI	TPO dose (μg)	N	red cells x 10^{12}/l	white cells x 10^{9}/l	platelets x 10^{9}/l
-	-	8	7.1 ± 0.6	0.4 ± 0.2	156 ± 68
+2 h	0.3	12	9.1 ± 0.6	1.1 ± 0.3	742 ± 92
-2 h	0.3	3	9.0 ± 0.1	0.7 ± 0.2	532 ± 54
-2 h	30	3	9.0 ± 0.3	0.8 ± 0.2	718 ± 86
+24 h	0.3	15	7.5 ± 0.5	0.4 ± 0.1	465 ± 41
normal mice	-	19	10.2 ± 0.6	5.3 ± 1.9	1,123 ± 89

Experiment design 2: BCBA F1 mice were subjected to 6 Gy total body irradiation (TBI) at time -2 d, -1 d, 0 and 0.9 Microgram TPO/mouse was administered i.p. at various time points as indicated in the legend of Fig. 10, i.e., 2 hours before the first fraction of TBI, 2 hours after the last fraction of TBI, or 3 fractions of 0.3 micrograms TPO 2 h after each fraction of TBI.

To simulate a protracted form of cytoreductive treatment more similar to chemotherapy, TBI was given in three equal fractions of 3 Gy separated by 24 h each (experimental design 2). TPO was given in a total dose of 0.9 microgram as indicated in the legend of Fig. 10. The thrombocyte response was optimal when this dose of TPO was given in three equal fractions of 0.3 microgram TPO, 2 h after each fraction of TBI. Similar effects were shown for red cells and white cells. Table 2 shows that in this experimental setting also, a very high dose of TPO 2 h before the first fraction of TBI was equally effective. Fig. 11 and Fig. 12 show the hemopoietic progenitor cell data of bone marrow and spleen, respectively which demonstrate that the most optimal dose schedule for TPO, i.e., 3 x 0.3 microgram 2 h after each fraction of TBI, also rapidly normalized progenitor cell levels without major fluctuations seen in the other treatment groups.

Figure 13 shows the pharmacokinetic data following three doses of 0.3 microgram of TPO or a single dose of 0.9 microgram. Peak levels relevant occur about 2 h after i.p. injection. An effective level is 30 ng/ml plasma. However, the minimum effective TPO level has not yet been determined by titration experiments.

From the data, it can be seen that maintaining a high level of TPO during cytoreductive treatment results in multilineage stimulation and peripheral blood cell recovery.

Table 2 - Major peripheral blood cell counts 10 days after 3 x 3 Gy TBI and TPO administration

TPO time relative to TBI	TPO dose (µg)	N	red cells x 10^{12}/l	white cells x 10^{9}/l	platelets x 10^{9}/l
-	-	9	5.8 ± 0.8	0.3 ± 0.1	51 ± 25
3x +2 h	0.3	6	8.6 ± 0.2	1.0 ± 0.2	883 ± 163
+2 h	0.9	3	6.6 ± 0.7	0.4 ± 0.1	527 ± 135
-2 h	0.9	3	7.2 ± 0.2	0.5 ± 0.1	215 ± 80
-2 h	30	3	8.4 ± 1.0	0.9 ± 0.1	791 ± 156
normal mice	-	19	10.2 ± 0.6	5.3 ± 1.9	1123 ± 89

TPO appeared to be highly effective in mice exposed to a total dose of 9 Gy TBI in three equal fractions separated by 24 h each, if administered intraperitoneally 2 h after each TBI fraction. TPO administration prevented the severe reduction of thrombocyte numbers observed in the placebo control group, stimulated the recovery of granulocytes and also fully prevented the development of anemia. Similar to the 6 Gy single TBI experiments, the effect appeared to be mediated by accelerated reconstitution of progenitor cells of multiple blood cell differentiation lineages. This implies that the efficacy of TPO is dependent on residual immature multilineage cells, which need to be stimulated by TPO in a time window following TBI.

We approached the question as to whether immature cells were involved by simple short-term quantitative transplantation assay. Following transplantation of bone marrow into lethally irradiated recipients, the number of spleen colonies at day 13 (CFU-S-13) is a meausure for relatively immature repopulating stem cells associated with the initial short-term wave of hemopoietic reconstitution, which lasts for several months. By this assay, the effect of TPO treatment on immature progenitor cells could be directly demonstrated. In mice exposed to 9 Gy TBI in three equal fractions, the number of detectable CFU-S-13 of TPO treated mice were approximately 14-fold increased at 24 h after the last fraction of TBI compared to the placebo control mice (Table 3). Similar increases were observed in progenitor cell numbers along the three lineages examined. These results provided a strong indication of a major effect of TPO on the most immature multilineage cells detectable by a clonogenic assay, consistent with the presence of TPO receptors on such immature cells.

The small time window to achieve optimal efficacy of TPO is peculiar in view of the slow phase of the wash-out of TPO levels, which has a terminal half-life of approximately 20 h and would result in approximately similar levels at all time points within the first twelve h after administration with the exception of the initial rise due to the distribution in plasma. This led us to speculate that the high levels of TPO in the first few hours after administration would be of decisive importance. The

latter hypothesis was tested in two ways, i.e., by pharmacokinetic measurements and by administration of a very high dose of TPO before TBI to examine whether an efficacy could be reached similar to that obtained by TPO administration after each fraction of radiation. By superimposing the pharmacokinetic data on the efficacy data, it can be derived that effective levels are larger than 20 ng/ml and occur approximately 2 h after i.p. administration. From the latter observation, we concluded that in these mice a high level of TPO approximately 4 h after TBI is required to alleviate the radiation induced bone marrow syndrome by stimulation of immature target cells. This was directly tested by administration of a dose of 30 micrograms TPO i.p. (calculated on the basis of the initial distribution) 2 h before the first radiation fraction. The results demonstrated that indeed such very high levels of TPO during the fractionated TBI regime are decisive to prevent thrombocytopenia. Thrombocyte counts 10 days after the last dose of TBI in mice treated with 30 micrograms before the first dose of TBI were not significantly differently from those of the mice treated with the most effective schedule of 0.3 microgram TPO 2 h after each TBI fraction. A similar efficacy was obtained by the high dose of TPO 2 h before a single TBI dose of 6 Gy.

Table 3: Effects of TPO treatment following 9 Gy TBI (3x3Gy, 24 hours apart) on CFU-S day 13 and progenitor cell content of bone marrow 24 hours after the last dose of TBI in mice treated with TPO 2 hours after each dose of TBI vs control

Treatment	CFU-S d13 (colonies per femur)	GM-CFU /femur	BFU-E /femur	CFU-Meg /femur
Placebo	1.9	597 ± 229	8.3 ± 3	22 ± 10
TPO 3*0.3	27.3	2026 ± 131	558 ± 282	430 ± 135

EXAMPLE 10 - Study of Patients receiving rhTPO and undergoing Autologous Bone Marrow Transplant (ABMT).

After harvest of the bone marrow harvest, all patients received cyclophosphamide 180 mg/kg, thiotepa 900 mg/m^2, ± carboplatin 600 mg/m^2. After infusion of the unmanipulated graft (day 0), patients began IV rhTPO on day 1, either qd or q3d, until either day 21 or a patient count ≥ 50 k/μl (see Table below). The rhTPO was administered to patients cohorts in dose-escalating fashion. All patients received G-CSF until the absolute neutrophil count was ≥ 500/μl. Platelets were transfused for a platelet count < 20 K/μl, or as clinically indicated.

Platelet recovery was defined as the first day of an unsupported platelet count ≥ 25,000/μl (simple definition) or, more rigorously, the first of ≥ 2 consecutive days of untransfused platelet counts ≥ 25,000/μl with the second count being greater than or equal to the preceding value (stable or rising definition). Neutrophil recovery was defined as the first of 2 consecutive days with an absolute

neutrophil count ≥ 500/μl. Patients were compared to 15 historical controls undergoing autologous bone marrow transplant using the same transplant regimens.

Table

Dose Level	Number (N)	Median Number of Platelet Transfusions (range)	Median Day of Platelet Recovery (range)	Median Day of Neutrophil Recovery (≥500/ml) (range)
1 (0.3 μg/kg)	7	6 (2-11)	18 (11-25)	10.5 (9-13)
2 (0.6 μg/kg)	6	7 (3-15)	16.5 (12-40)	11 (10-28)
3 (1.2 μg/kg)	7	6 (3-11)	21 (15-28)	11 (10-13)
4 (2.4 μg/kg)	3	11 (6-16)	24.5 (16-33)	15.5 (11-20)
5 (0.6 μg/kg)	3	6 (5-10)	17 (15-21)	11 (10-11)
6(4.8 μg/kg)	6	8 (4-18)	19 (14-34)	11.5 (10-14)
All Study Patients	32	6 (2-18)	18 (11-40)	11 (9-28)
Historical Controls	15	Not Available	19	10

rhTPO in all Dose Levels was administered Q3D with the exception of Dose Level 5 where the rhTPO was administered QD.

Thirty-three patients with a median age 49 years (23-59 years), were enrolled in this study; all evaluable for safety and hematologic response. Seven patients received rhTPO at the 0.3μg/kg dose level, 7 at the 0.6 μg/kg level; 7 at 1.2 μg/kg; 3 at 2.4 μg/kg; and 6 at 4.8 μg/kg (see Table above). All levels were safely tolerated, and rhTPO has not been associated with any study-drug-related adverse events. Chemotherapy-related adverse effects occurred as expected. One patient experienced prolonged pancytopenia due to human herpes virus-6 infection. Only two hemorhagic episodes during the period of thrombocytopenia, epistaxis and a subdural hematoma, were observed, both when platelet counts were < 25,000μL. Both resolved without long-term sequelae. There were no episodes of thrombosis or veno-occluusive disease noted. Over the entire group of patients in this study, the median time to plt recovery (stable and rising definintion) was 17.5 days.

In summary, rhTPO in combination with G-CSF appears to be safe and well-tolerated in patients undergoing myeloablative therapy and rescue with autologous bone marrow.

EXAMPLE 11 - Peripheral Blood Progenitor Cell (PBPC) mobilization study in Patients with Breast Cancer

Patients were treated in cohorts of 3 evaluable patients at a thrombopoietin (TPO) dose of 0.6,1.2 or 2.4 μg/kg IV with dose escalation in the successive cohorts if toxicities related to

thrombopoietin did not occur. No serious toxicity related to TPO occurred. The optimal biologic dose is considered the lowest dose which will maximize CD34+ cells/kg/liter of blood processed in the PBSC collection. Secondary endpoints are the intervals to recovery of granulocytes and platelets after CVP and after CBT/PBPC transplant.

Treatment Remimen

CVP: Cyclophosphamide 1.5 mg/m^2/d IV+ mesna; etoposide 250 mg/m^2 dl-3; cisplatin 40 mg/m^2 dl-3, followed by single dose thrombpoietin 0.6-2.4 μg/kg IV d4 G-CSF 6μg/kg q12h.

Upon recovery of WBC > 1.0, PBPC collection by large volume apheresis 3x BV, target > 3 x 10^6 CD34+ cells/kg.

CBT: Cyclophosphamide2.0 gm/m^2 IV, Thiotepa 240 mg/m^2/d, BCNU 150 mg/m^2/d days -8, -7, -6 with reinfusion of the cryopreserved cells on day 0.

G-CSF 5 μg/kg/d SC until recovery of granulocytes.

Table

Parameters	G-CSF only	G-CSF + TPO	Independent t-test against null hypothesis no difference at α.05
Apheresis Collection Parameters (mean ± std)			
Patient Population	6 patients 4/6 (67%) single collection 2/6 (33%) two collections	12 patients 10/12 (83%) single collections 2/12 (17%) two collections	n/a
Patient Weight (kg)	71.5 ± 12.0	72.5 ± 15.1	p = .4908
Blood Processed (L) per Apheresis	13.298 ± 1.255	13.300 ± 1.825	p = .3626
Collection Efficiency of CD34 cells	nd	60% (median)	nd
Transplant Dose (10^6) per kg (mean ± std)			
CD34$^+$	15.268 ± 18.867	34.479 ± 32.235	p = .1999
CD34$^+$ THY$^+$	5.653 ± 7.267	23.417 ± 22.411	p = .0802
CD34$^+$ THY^{dim}	4.842 ± 6.447	13.671 ± 14.941	p = .1900
CD34$^+$ CD41$^+$	2.511 ± 3.107	1.592 ± 1.345	p = . 3864
CD34$^+$ CD41$^+$ THY$^+$	0.537 ± 0.383	0.753 ± 0.639	p = .4617
CD34$^+$ CD41$^+$ THY^{dim}	0.444 ± 0.364	0.450 ± 0.372	p = . 9713

Engraftment: days to reach cell target (mean ± std)			
ANC_{500}	10 ± 1	9 ± 1	nd
PLT_{20}	11 ± 2	9 ± 2	nd
PLT_{50}	12 ± 1	12 ± 3	nd

Thrombopoietin was well tolerated when given with G-CSF following CVP chemotherapy for PBPC mobilization.

Mobilization was enhanced compared to historical controls. A median of one apheresis was required to reach target cell dose. Hematopoietic recovery post transplant was rapid.

EXAMPLE 12 - PBPC Study of Patients with Breast Cancer

A phase I clinical trial was conducted to assess the feasibility and possible efficacy of single and repeated doses of thrombopoietin (TPO) together with G-CSF 10 microgram/kg for peripheral blood progenitor cell (PBPC) mobilization followed by high dose chemotherapy (HDCT) with cisplatin, VP-16 and cyclophosphamide (Cy) in patients with high-risk and responsive stage IV breast cancer.

The HDCT treatment scheme was as follows:

Day -12	Cisplatin	125 mg/m^2
	VP-16	30 mg/kg
Day -5	Cisplatin	125 mg/m^2
	VP-16	30 mg/kg
Day -3	Cy	100 mg/kg
Day -2	25% of	PBPC (transplant)
Day 0	75% of	PBPC (transplant)

The TPO dosing scheme was as follows:

	Day	TPO Dose	# of Patients
Arm A	1	0.3 microgram/kg	3
		1.2 microgram/kg	3
		2.4 microgram/kg	4
Arm B	-3, -1, 1	0.6 microgram/kg	6
		1.2 microgram/kg	0
		0.3 microgram/kg	3

Mobilization consisted of TPO and G-CSF 10 microgram/kg (5 microgram/kg BID) in group A. G-CSF 10 microgram/kg once a day in group B. G-CSF 10 microgram/kg (5 microgram/kg BID) in group C.

Apheresses were carried out processing ~ 10 liter of blood via a CS-3000 Fenwall or COBE-Spectra cell separator. PBPC-s were cryopreserved in a solution containing 5% DMSO and were frozen by simple immersion into a -130 °C freezer. Group A vs B vs C comparisons were performed using the Kruskal-Wallis test. Group A vs B were compared using Wilcoxon rank-sum test. Data on PBPC apheresis, hematopoietic recovery and transfusion requirements are presented as median (range).

Platelet (PLT) transfusions were provided for ≤ 20,000 PLT/ul or as clinically indicated. Platelet independence is defined as the day after the last platelet transfusion. Single pheresis products and pooled platelet products are counted as 1 PLT transfusion.

PATIENT CHARACTERISTICS

	Group A	**Group B**	**Group C**
Age	46 (34-61)	44 (28-62)	45 (36-57)
Prior Regimens	1 (1-4)	1.5 (1-5)	1 (1-2)
Prior ChemoRx cycles	6 (3-23)	5 (3-18)	4 (3-7)
	N (%)	**N (%)**	**N (%)**
Stage II/III	12 (73)	14 (54)	11 (100)
Stage IV	7 (37)	12 (46)	0
Prior Rt	3 (16)	6 (23)	0

COMPARISON OF APHERESIS REQUIREMENTS, $CD34^+$ and MNC YIELD

Group	A	B	C	p-value
N	19	26	11	
Apheresses	2 (2)	4 (2-6)	3 (3-5)	.0001
MNC*	4.1 (.5-6.5)	2.3 (1.2-4.3)	NA	.0001
$CD34^+$	5 (2-12.7)	0.9 (0.1-4.3)	2.4 (0.4-7-7)	.0001

* Mononuclear cell yield in 10^8/kg; $^+$ CD34 yield in 10^6/kg

HEMATOPOIETIC RECOVERY and TRANSFUSION REQUIREMENTS

Group	A	B	p-value
N	14	26	
AGC ≥ 500/ul	7.5 (6-9)	8 (7-11)	.0001
PLT independence	9 (7-11)	10.5 (5-21)	.0001
PLT transfusions	4 (1-10)	6 (2-52)	.002
RBC transfusions	3 (2-7)	4 (2-6)	.009

RBC = red blood cell; AGC =

TPO in the dose ranges tested is well tolerated. TPO in combination with G-CSF increases the efficacy of PBPC mobilization and $CD34^+$ yield. TPO and G-CSF mobilized PBPC accelerate both platelet and granulocyte recovery.

EXAMPLE 13 - Other Embodiments

The invention specifically contemplates treatment cycles in which the radiation or chemotherapy agent is administered on multiple consecutive days, for example on 4, 5, 6 or 7 consecutive days, where the TPO dose is administered prior to the first of the consecutive days and/or concurrent with one or more of the consecutive days of the treatment cycle. For a treatment cycle of 5 consecutive days, TPO might be given on day -1 and on days 6, 9, 12, 15, etc. In another example, for a treatment cycle of 7 consecutive days, TPO might be given on day -1 and on days 2, 4, 6, 8, 10, 12, etc. or on day -1 and on days 4, 6, 8, 10, 12, etc. In another embodiment, the radiation or chemotherapy agent will be given on alternate days of the treatment cycle, e.g. days 1, 3, 5. In this embodiment, TPO might be given on day -1 and on days 2, 4, 6, 8, 10, etc.

EXAMPLE 14

A further use of TPO according to the invention is for *ex vivo* expansion of progenitor cells obtained, for example, from mammalian bone marrow, peripheral blood, or umbilical cord blood. Progenitors expanded in such a manner can be used for allogeneic stem cell transplant for patients who have been treated with chemotherapy or radiation treatment. A progenitor population that is enriched for stem cell activity is the $CD34^+$ population and this population can be futher enriched by selecting for $CD34^+CD38^-$ cells. This population has the ability to generate multilineage hematopoietic colonies in vitro and multipotential hematopoietic engraftment in vivo. Progenitor stem cells for expansion can be obtained from peripheral blood by pheresis and from bone marrow aspirates by standard techniques. Hematopoietic progenitors can also be obtained from umbilical cord blood. Hematopoietic stem cells having the $CD34^+$ phenotype may then be isolated from the blood or marrow using an immunomagnetic enrichment column. The isolated cells may then be cultured in a cell growth medium

containing a cocktail of the growth factors TPO, Flt-3 and c-kit ligand in order to expand or increase the number of cells in the stem cell population. The growth factors are added in amounts sufficient to stimulate growth of the progenitor stem cells. Preferably, each of the growth factors is added in an amount of about 10 to about 100 ng/ml to growth media containing about 10^2 to about 10^6 stem cells/ml. The cells are cultured using standard culture techniques, for example, about 35 - 40°C in aproximately 5% CO_2 for about 1 - 8 weeks. The cultures are exchanged into fresh media containing growth factors every week. The growth media may contain other conventional nutrients, fetal serum, etc in standard amounts. The expanded cells are then readministered as an allogeneic stem cell transplant according to known procedures.

In the following table is shown the ability of the $CD34^+$ cultures after 8 weeks expansion to generate lymphohematopoiesis in vivo using the SCID-hu bone assay. In bone grafts injected with cells from the expanded cultures these cells contributed to the lymphoid, myeloid and progenitor hematopoietic compartments. This shows that the expanded cells maintained their in vivo multipotent engraftment potential.

TABLE: Engraftment of TPO/KL/FL expanded progenitors in SCID-hu mice.

Condition	Study	% of mice engrafted	% donor HLA/CD34	% donor HLA/CD33	% donor HLA/CD19
TPO/FL/KL	1	80%	4.9 +/- 1.5	8.8 +/- 2.1	55.3 +/- 8.9
TPO/FL/KL	2	100%	6.9 +/- 2.4	4.9 +/- 1.9	59.6 +/- 14.1

MATERIALS AND METHODS

Isolation of human hematopoietic stem cell population: Hematopoietic progenitor cell populations were isolated from human bone marrow. Briefly, the mononuclear fraction was enriched for $CD34^+$ cells using animmunomagnetic enrichment column. (Miltenyi Biotech, Auburn, CA). Purity was routinely >90% by FACS.

Suspension culture assays: Hematopoietic stem cell populations were seeded at 2e4 cells/mL in IMDM Gibco BRL (Grand Island, NY), plus 10 % fetal bovine serum (Gibco BRL), 10^{-5} M 2-mercaptoethanol, 10^{-6} M hydrocortisone , and 2 mM L-glutamine (Gibco BRL). Growth factors were added at the following concentrations: Flt-3 ligand (Immunex, Seattle WA) 50 ng/mL, TPO (Genentech, S. San Francisco, CA) 50 ng/mL, and c-kit ligand (R and D Systems) 50 ng/mL. Cultures were incubated at 37°C/5% CO_2 for seven days. On day 7 all wells were harvested and all cells were

counted by hemacytometer. For subsequent platings, 2e4 cells/mL were added to fresh media and growth factors and incubated for an additional seven days. All conditions were done in duplicate.

Flow cytometric analysis: For FACS analysis, cells were resuspended in PBS/2% FBS at 1e6 cells /mL and stained with mouse anti human CD34 FITC, CD38 PE (Becton Dickenson). Viable cells were selected by propidium iodide exclusion and analyzed on a FACscan (Becton Dickenson).

Colony Assays: Methylcellulose colony assays were performed using "complete" myeloid methylcellulose media (Stem Cell Technologies, Vancouver, B.C.). Cells were seeded in methylcellulose at 1,000 cells/mL and plated in 4 x 35 mm gridded dishes. Colonies were counted and visually phenotyped on an inverted phase contrast microscope after 14 days in culture.

SCID-hu mouse reconstitution assay: CB-17 *scid/scid* mice were implanted with fetal bone marrow as described previously. Mice were used so that the grafts and cells were mismatched for major histocompatibility complex (MHC) class I antigens. Mice received 250 rads whole body γ irradiation and then followed by injection of 30,000 cultured human bone marrow cells into the bone graft. Eight weeks after the injection of the cells, the bone grafts were harvested and analyzed for donor HLA contribution to FITC conjugated anti human CD34 (progenitor), anti human CD33 (myeloid), and anti human CD19 (lymphoid). Donor HLA positive cells were then sorted by FACS. Thirty thousand donor HLA positive cells were then injected into secondary recipients in the same manner as the primary recipients. Eight weeks after injection the secondary bone grafts were removed and analyzed for engraftment of CD34, CD19, and CD33.

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed such specific methods, those skilled in the art will well enough know how to devise alternative reliable methods at arriving at the same information in using the fruits of the present invention. Thus, however detailed the foregoing may appear in test, it should not be construed as limiting the overall scope thereof; rather, the ambit of the present invention is to be determined only be the lawful construction of the appended claims. All documents cited herein are hereby expressly incorporated by reference.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Novel Administration of Thrombopoietin

(iii) NUMBER OF SEQUENCES: 24

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(v) COMPUTER READABLE FORM:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCGATATCG ATCAGCCAGA CACCCCGGCC AG 32

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTAGCTCTA GACAGGGAAG GGAGCTGTAC ATGAGA 36

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCTAGATCTA GATCACCTGA CGCAGAGGGT GGACC 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCGATATCG ATAGCCAGAC ACCCCGGCCA G 31

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTCGACGTC GACGTCGGCA GTGTCTGAGA ACC 33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTCGACGTC GACTCACCTG ACGCAGAGGG TGGACC 36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCGTATGCC AGCCCGGCTC CTCCTGCTTG TGACCTCCGA GTCCTCAGTA 50

AACTGCTTCG TG 62

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTCACGAAG CAGTTTACTG AGGACTCGGA GGTCACAAGC AGGAGGAGCC 50

GGGCTGGCAT A 61

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC TTCTTAA 37

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGTTAAGA AGAAATGCGA TATTCTTTTT CATAATT 37

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC ATCACCATCA CCATCACCAT 50

CACATCGAAG GTCGTA 66

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACGACCTCG ATGTGATGGT GATGGTGATG GTGATGAAAT GCGATATTCT 50

TTTTCATAAT TCCG 64

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC ATCACCATCA CCATCACCAT 50

CACATCGAAC CACGT 65

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TACGTGGTTC GATGTGATGG TGATGGTGAT GGTGATGAAA TGCGATATTC 50

TTTTTCATAA TTCCGA 66

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCACCCTCT GCGTCAGGT 19

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCTACCTGA CGCAGAGG 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCAGCAGTTC TAGAATTATG TCNCCNGCNC CNCCNGCNTG TGACCTCCGA 50

ACACTGGAGG CT 62

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAAGGACATG GGAGTCACGA AGCAGTTTAC TGAGAACAAA TGACTCTTG 49

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTAGAATTAT GAAAAAGAAT ATCGCATTTA TCGAAGGTCG TAGCC 45

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TACGACCTTC GATAAATGCG ATATTCTTTT TCATAATT 38

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC TTCTTAAACG TAGCC 45

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TACGTTTAAG AAGAAATGCG ATATTCTTTT TCATAATT 38

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 332 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu
1 5 10 15

Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro
20 25 30

Glu Val His Pro Leu Pro Xaa Pro Val Leu Leu Pro Ala Val Asp
35 40 45

Xaa Xaa Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala
50 55 60

Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met
65 70 75

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu
80 85 90

Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln
95 100 105

Ser Leu Leu Gly Thr Gln Xaa Xaa Xaa Xaa Gly Arg Thr Thr Ala
110 115 120

His Xaa Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu
125 130 135

Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu
140 145 150

Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr
155 160 165

Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg Thr Ser Gly
170 175 180

Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr Thr Gly Ser
185 190 195

Gly Leu Leu Lys Xaa Gln Gln Gly Phe Arg Ala Lys Ile Pro Gly
200 205 210

Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr

215 220 225

Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe
230 235 240

Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser
245 250 255

Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly
260 265 270

Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu
275 280 285

Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu His
290 295 300

Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser
305 310 315

Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln
320 325 330

Glu Gly
332

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 153 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu
1 5 10 15

Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro
20 25 30

Glu Val His Pro Leu Pro Xaa Pro Val Leu Leu Pro Ala Val Asp
35 40 45

Xaa Xaa Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala
50 55 60

Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met
65 70 75

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu
80 85 90

Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln
95 100 105

Ser Leu Leu Gly Thr Gln Xaa Xaa Xaa Xaa Gly Arg Thr Thr Ala
110 115 120

His Xaa Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu
125 130 135

Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu
140 145 150

Cys Val Arg
153

WHAT IS CLAIMED IS:

1. A method for treating a mammal having or at risk for thrombocytopenia, comprising administering to a mammal in need of such treatment a single or low-multiple daily dose of a therapeutically effective amount of a thrombopoietin (TPO).
2. The method of Claim 1, wherein the mammal receives at least one treatment cycle of radiation and/or chemotherapeutic agent, the treatment cycle having a first treatment time T_0 and a last treatment time T_F for administering radiation and/or chemotherapeutic agent.
3. The method of Claim 2, wherein the dose is administered at T_0 plus or minus 24 hours.
4. The method of Claim 3, wherein the dose is administered at T_0 plus or minus ten hours.
5. The method of Claim 4, wherein the dose is administered at T_0 plus or minus six hours.
6. The method of Claim 5, wherein the dose is administered at T_0 plus or minus two hours
7. The method of Claim 2, wherein the dose is administered at T_0 or prior to T_0, but not more than seven days prior to T_0.
8. The method of Claim 7, wherein the dose is administered prior to T_0, but not more than one day prior to T_0.
9. The method of Claim 2, wherein $T_0 = T_F$.
10. The method of Claim 2, wherein the treatment cycle comprises multiple treatments of radiation and/or chemotherapeutic agent.
11. The method of Claim 10, wherein the dose is administered prior to T_F, but not more than seven days prior to T_F.
12. The method of Claim 11, wherein the treatment cycle comprises 2-10 treatments.
13. The method of Claim 2, further comprising administering a second dose of TPO after T_0.
14. The method of Claim 13, wherein the second dose is administered after T_F.
15. The method of Claim 14, wherein the second dose is administered not more than 24 hours after T_F.
16. The method of Claim 2, further comprising administering a dose of TPO concurrent with a treatment time.
17. The method of Claim 2, wherein the mammal receives multiple treatment cycles.
18. The method of Claim 21, wherein the mammal receives 2-6 treatment cycles.
19. The method of Claim 1, wherein the TPO is administered in a single therapeutically effective daily dose.

20. The method of Claim 1, wherein the TPO is administered in a low-multiple therapeutically effective daily dose.

21. The method of Claim 20, wherein the low-multiple dose comprises 2-6 doses per day.

22. The method of Claim 1, further comprising co-administering a therapeutically effective amount of an agent selected from the group consisting of a cytokine, a colony stimulating factor and an interleukin.

23. The method of Claim 22, wherein the agent is selected from the group consisting of KL, LIF, G-CSF, GM-CSF, M-CSF, EPO, FLT-3, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 and IL-11.

24. The method of Claim 1, wherein the dose is administered together with a carrier or excipient.

25. The method of Claim 24, wherein the carrier or excipient contains a chelating agent.

26. The method of Claim 25, wherein the chelating agent is EDTA.

27. The method of Claim 1, wherein the TPO is selected from the group consisting of

a) a TPO fragment polypeptide;

b) a TPO isoform polypeptide;

c) a TPO chimeric polypeptide; and

d) a pegylated TPO polypeptide.

28. The method of Claim 27, wherein the pegylated polypeptide is prepared with polyethylene glycol.

29. The method of Claim 1, wherein the TPO is selected from the group consisting of

a) a TPO polypeptide that is isolated from a mammal;

b) a TPO polypeptide that is made by recombinant means; and

c) a TPO polypeptide that is made by synthetic means.

30. The method of Claim 1, wherein the TPO is selected from the group consisting of

a) a polypeptide that is human; and

b) a polypeptide that is non-immunogenic in a human.

31. The method of Claim 1, wherein the TPO is represented by the formula:

X-hTPO(7-151)-Y

where hTPO(7-151) is a human TPO (hML) amino acid sequence from Cys^{7} through Cys^{151} inclusive; X is the amino group of Cys^{7} or one or more of the amino-terminus amino acid residue(s) of mature TPO or amino acid residue extensions thereto including Met, Lys, Tyr or amino acid substitutions thereof including arginine to lysine or thrombin); and Y is the carboxy terminal group of Cys^{151} or one or more carboxy-terminus amino acid residue(s) of mature TPO or extensions thereto.

32. The method of Claim 1, wherein the TPO is human TPO.

33. The method of Claim 32, wherein the TPO is human TPO(153).

34. The method of Claim 32, wherein the TPO is human TPO(332).

35. The method of Claim 32, wherein the TPO is $rhTPO_{332}$.

36. The method of Claim 1, wherein the TPO is administered intravenously.

37. The method of Claim 1, wherein the TPO is administered subcutaneously.

38. The method of Claim 1, wherein the TPO is administered by inhalation.

39. The method of Claim 1, wherein the dose is sufficient to maintain a blood level of TPO in the mammal of 35×10^{-12} M or greater during the treatment cycle.

40. The method of Claim 39, wherein the dose is sufficient to maintain a blood level of TPO of 100×10^{-12} M or greater during the treatment cycle.

41. The method of Claim 41, wherein the dose is sufficient to maintain a blood level of TPO of about $35\ 10^{-12}$ M to about 3500×10^{-12} M during the treatment cycle.

42. The method of Claim 1, wherein the dose ranges from about 0.1-10 microgram/kg of body weight.

43. The method of Claim 42, wherein the dose ranges from about 1 to about 5 microgram/kg of body weight.

44. The method of Claim 38, wherein the dose is about 5-1000 microgram/kg in an aerosol.

45. A method for increasing the number of CD34+ cells in a mammalian blood sample, comprising adding an effective amount of TPO and a cytokine selected from the group consisting of FLT-3, KL and a mixture thereof, to CD34+ cells in the sample.

46. The method of Claim 45, comprising adding TPO, FLT-3 and KL.

47. The method of Claim 45, further comprising administering the sample to a mammal.

48. The method of Claim 46, further comprising administering the sample to a mammal.

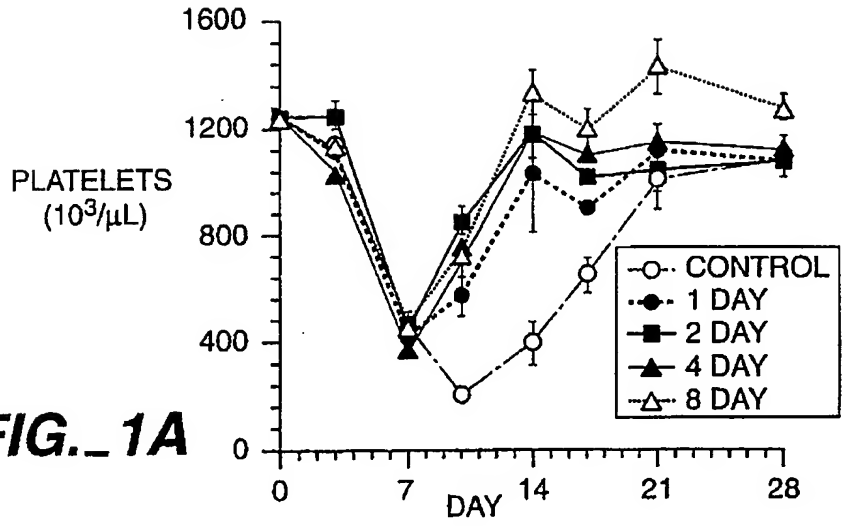


FIG._1A

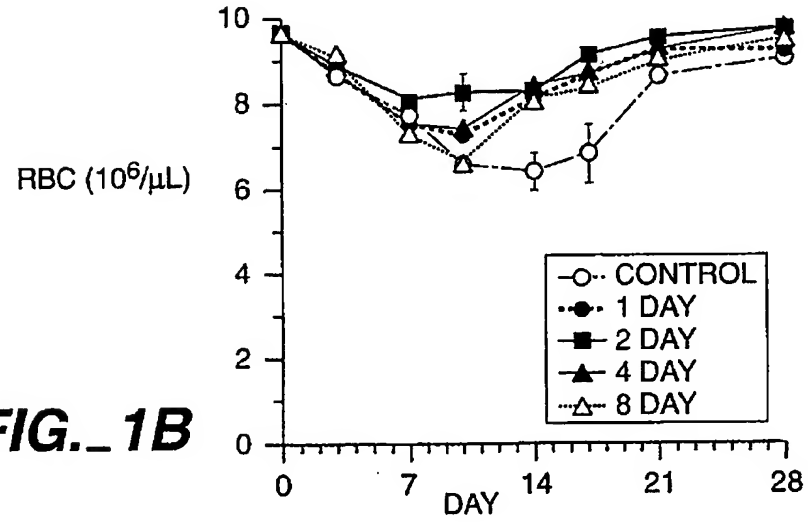


FIG._1B

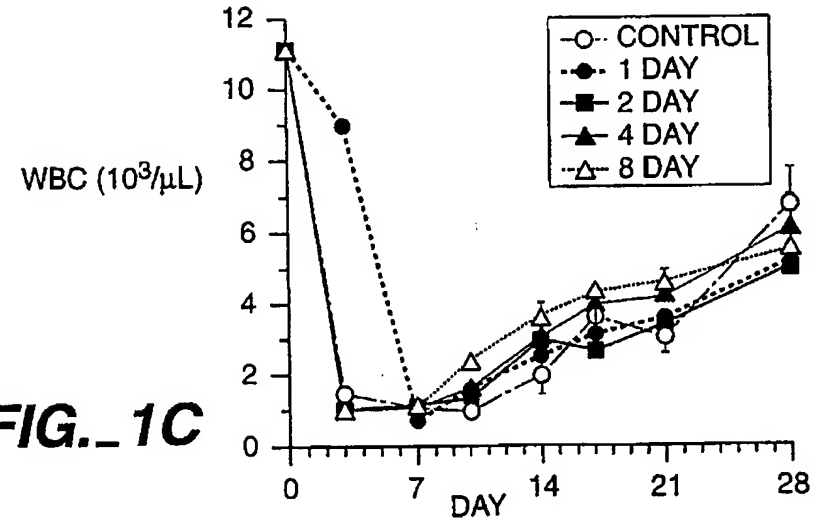


FIG._1C

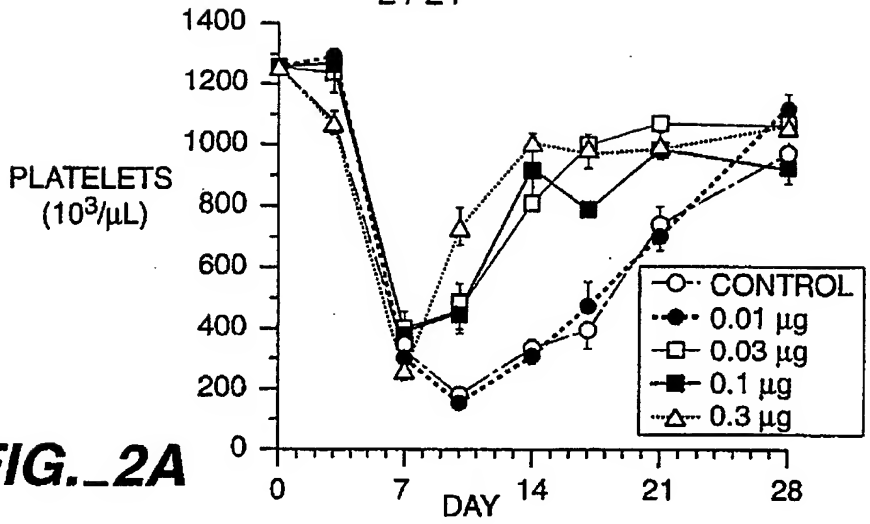


FIG._2A

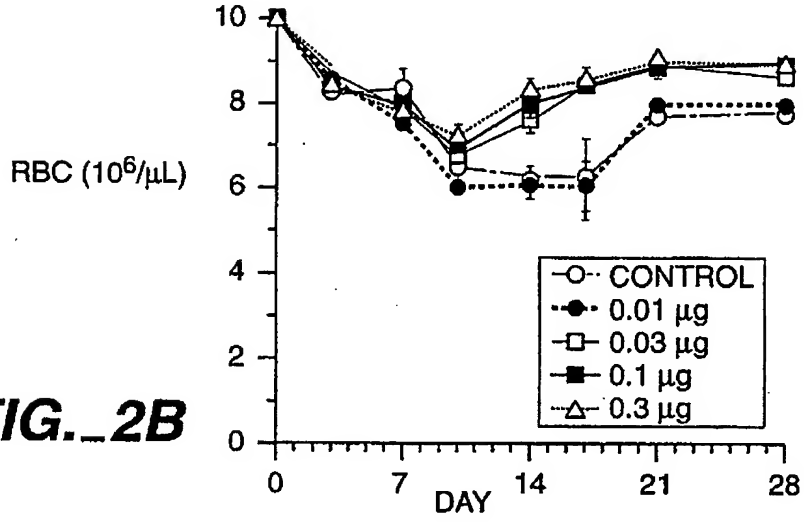


FIG._2B

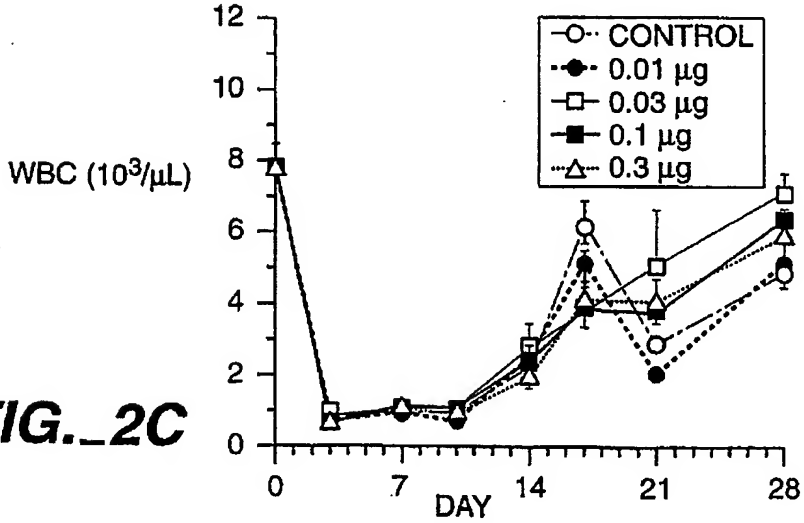


FIG._2C

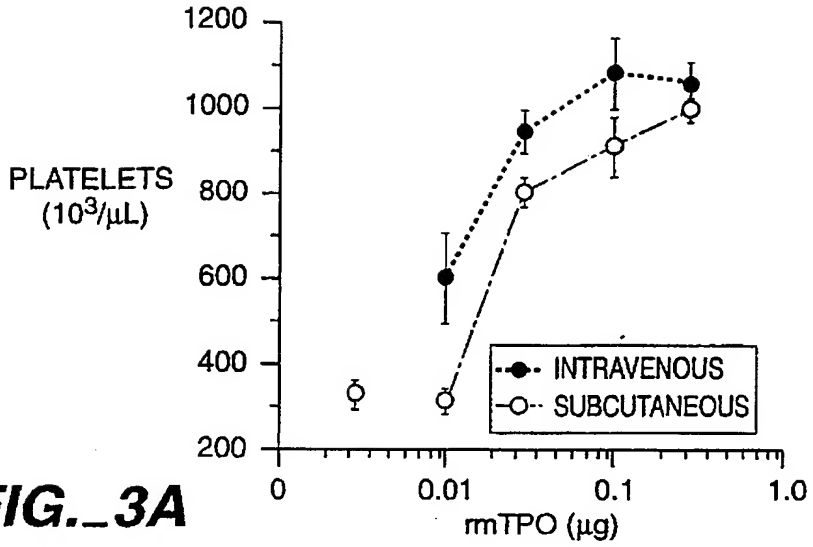

PLATELETS
(10^3/μL)
1200
1000
800
600
400
200
0
0.01
0.1
1.0
rmTPO (μg)
INTRAVENOUS
SUBCUTANEOUS

FIG._3A

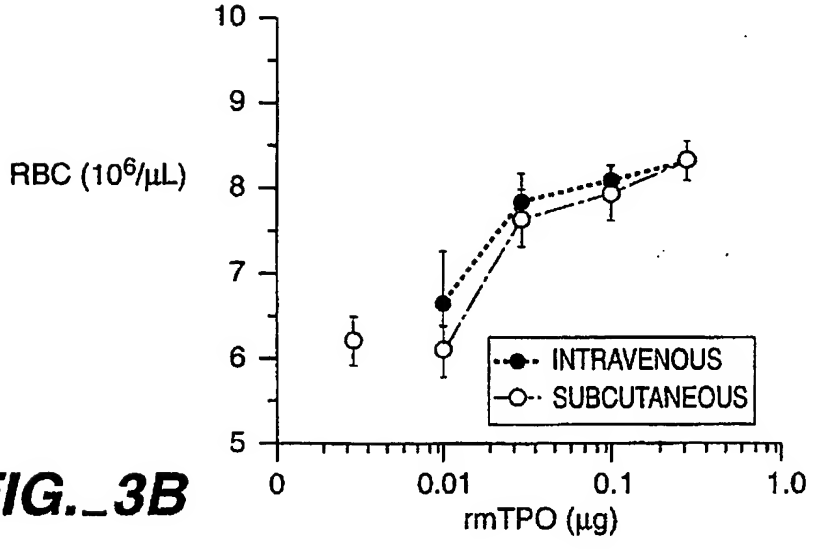

RBC (10^6/μL)
10
9
8
7
6
5
0
0.01
0.1
1.0
rmTPO (μg)
INTRAVENOUS
SUBCUTANEOUS

FIG._3B

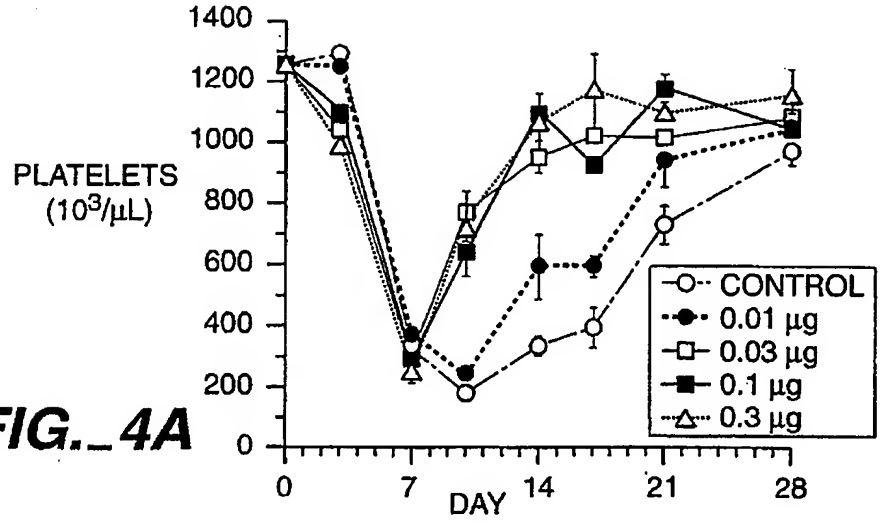


FIG._4A

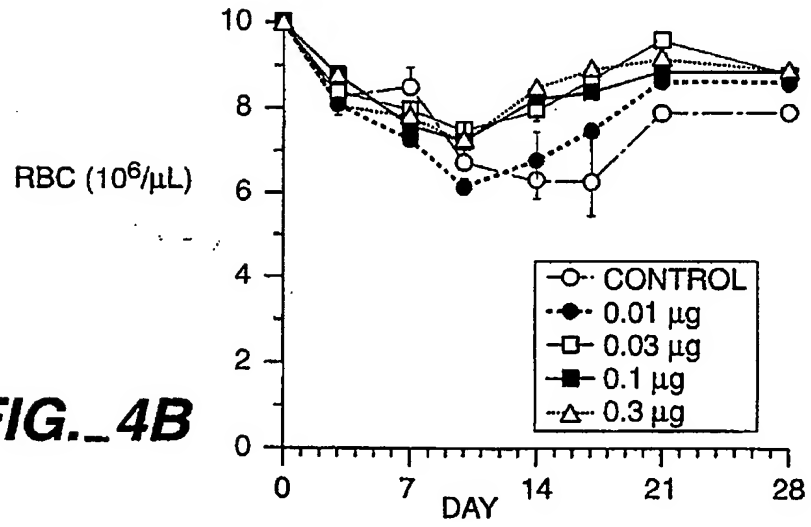


FIG._4B

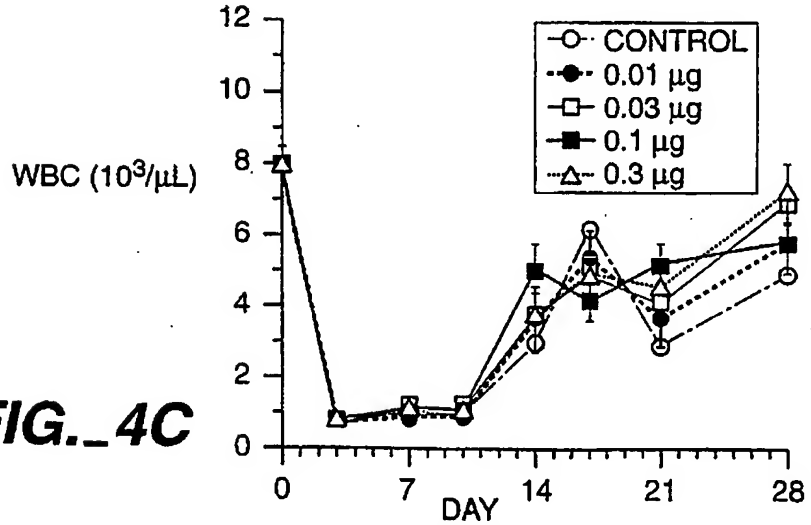


FIG._4C

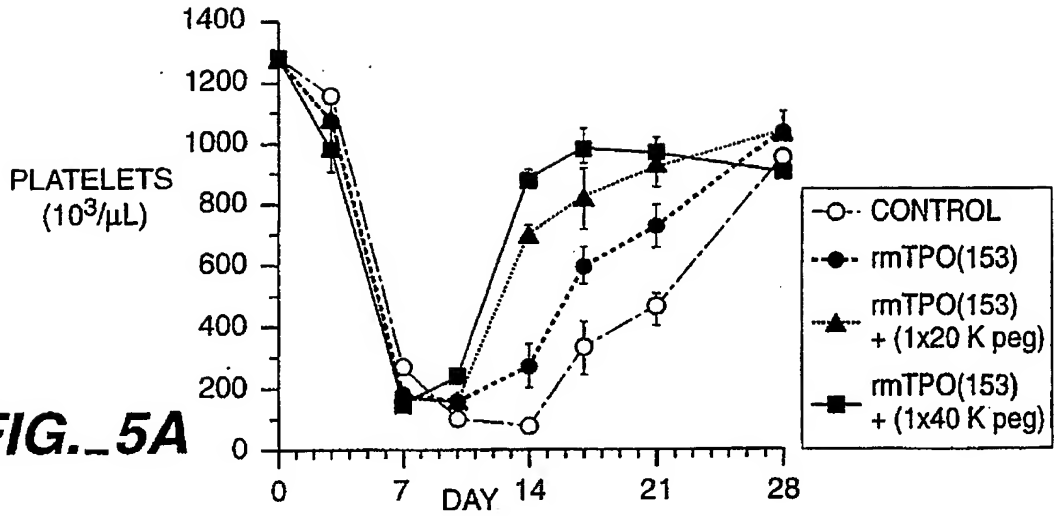


FIG._5A

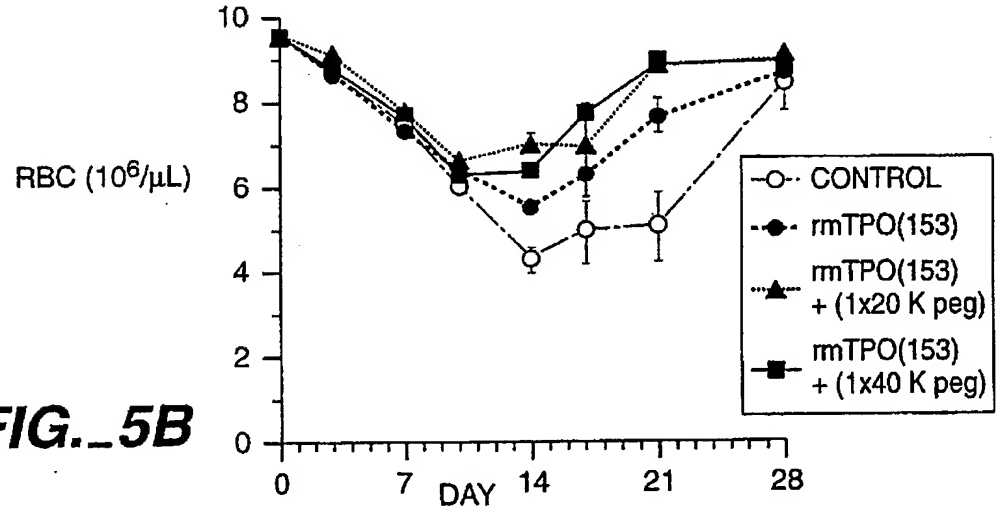


FIG._5B

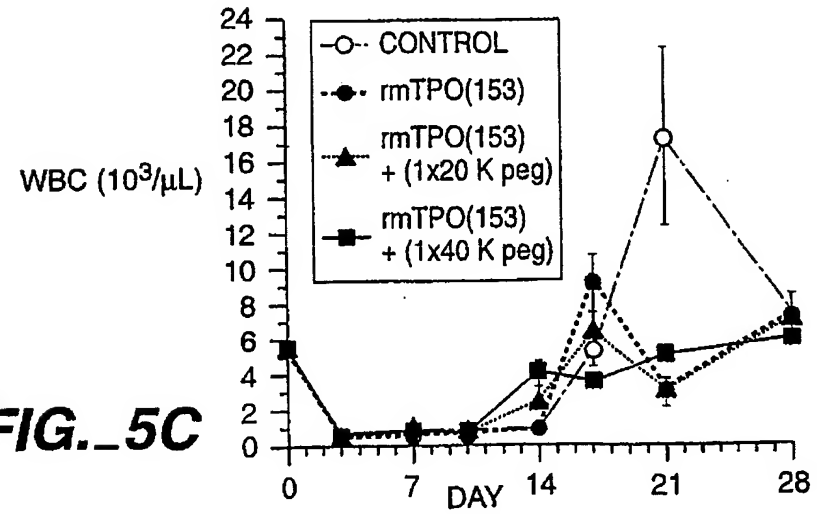


FIG._5C

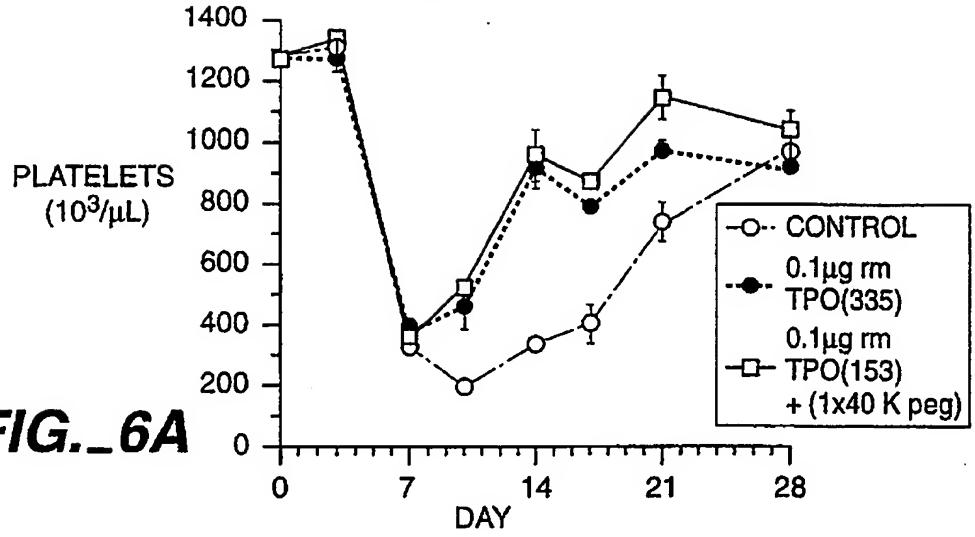


FIG._6A

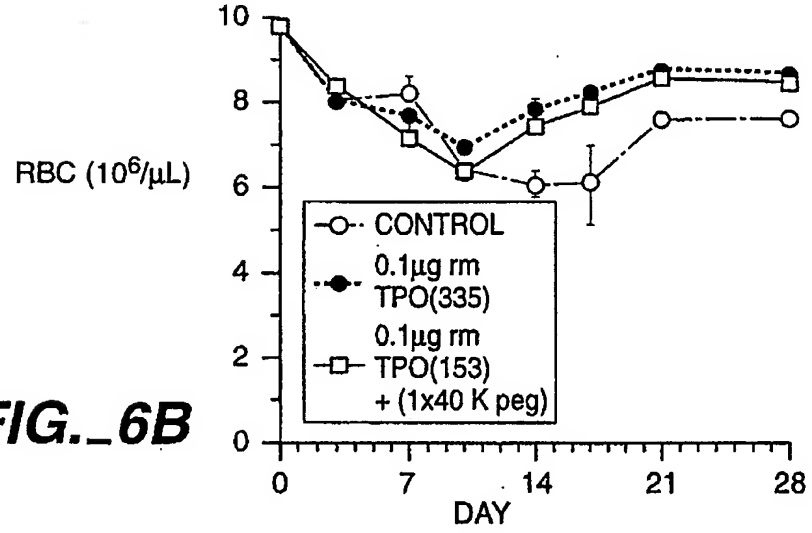


FIG._6B

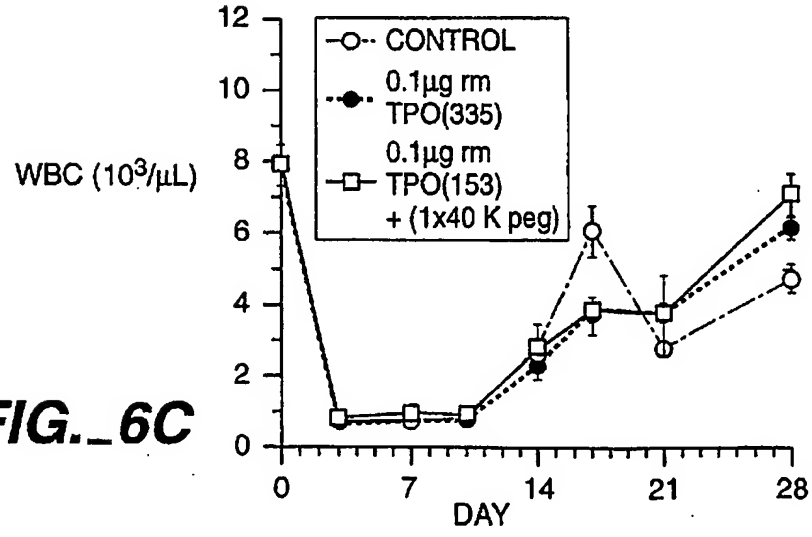


FIG._6C

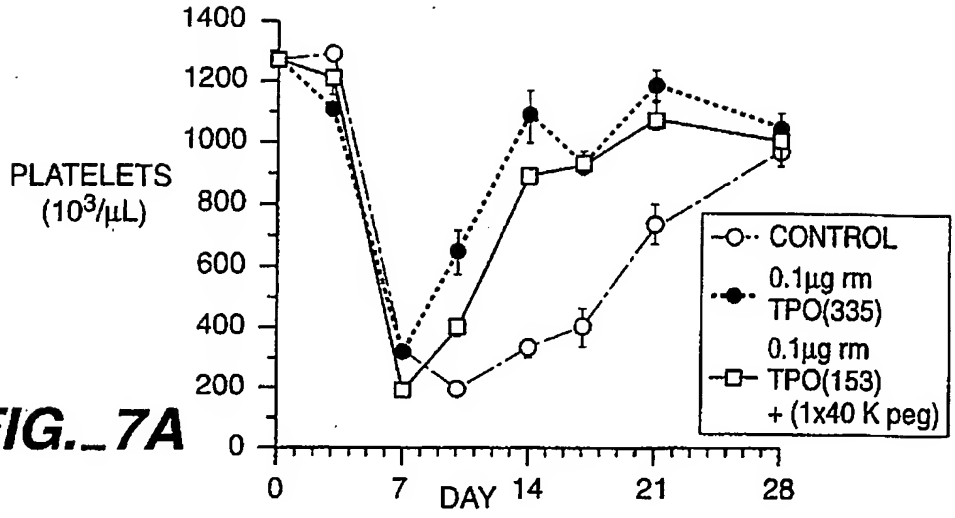


FIG._7A

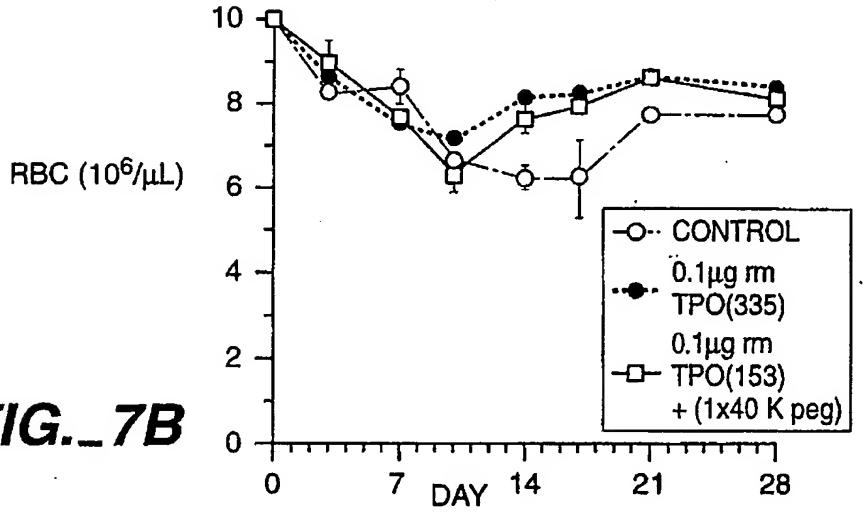


FIG._7B

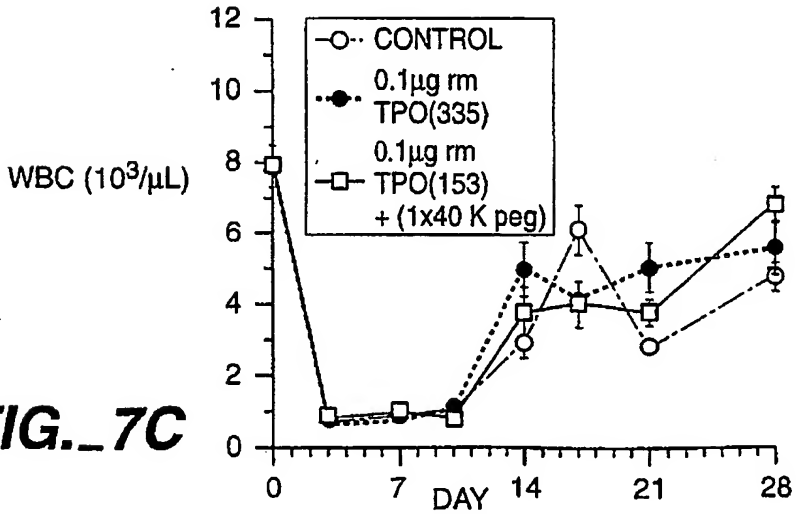


FIG._7C

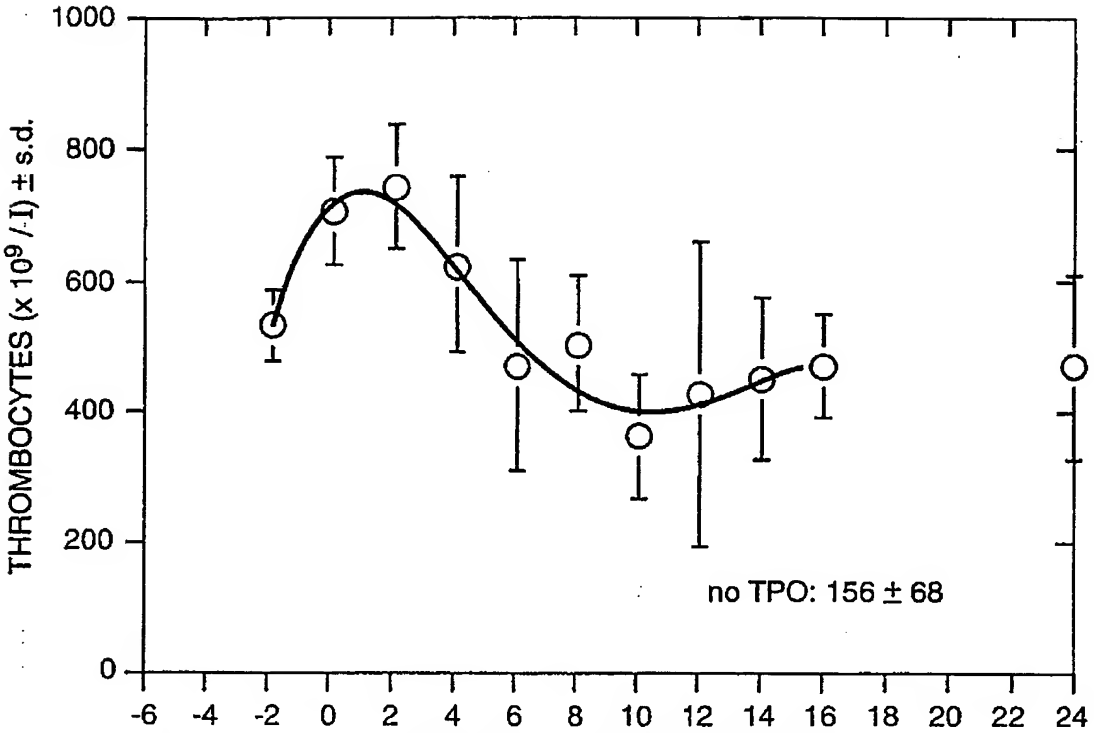

THROMBOCYTES (x 10⁹ /l) ± s.d.
1000
800
600
400
200
0
-6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24
no TPO: 156 ± 68
TPO (0.3 µg) ADMINISTRATION
(h AFTER TBI))

FIG._8

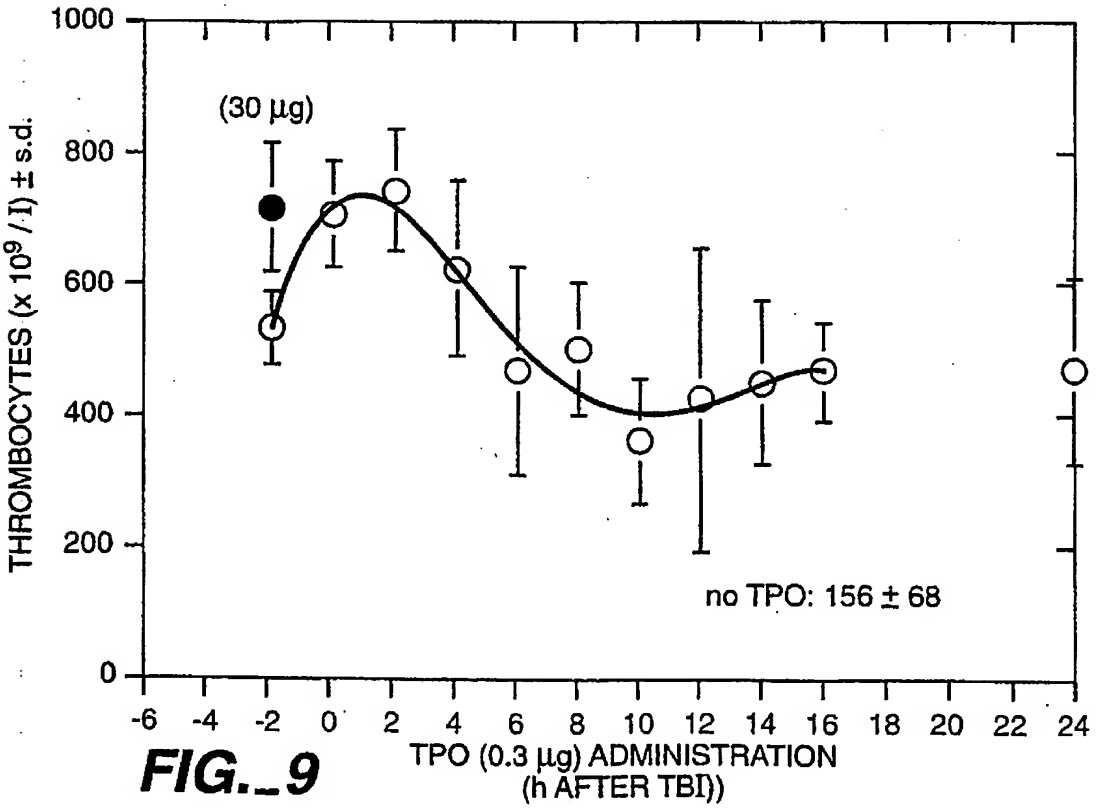

THROMBOCYTES (x 10⁹ /l) ± s.d.
(30 µg)
1000
800
600
400
200
0
-6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24
no TPO: 156 ± 68
TPO (0.3 µg) ADMINISTRATION
(h AFTER TBI))

FIG._9

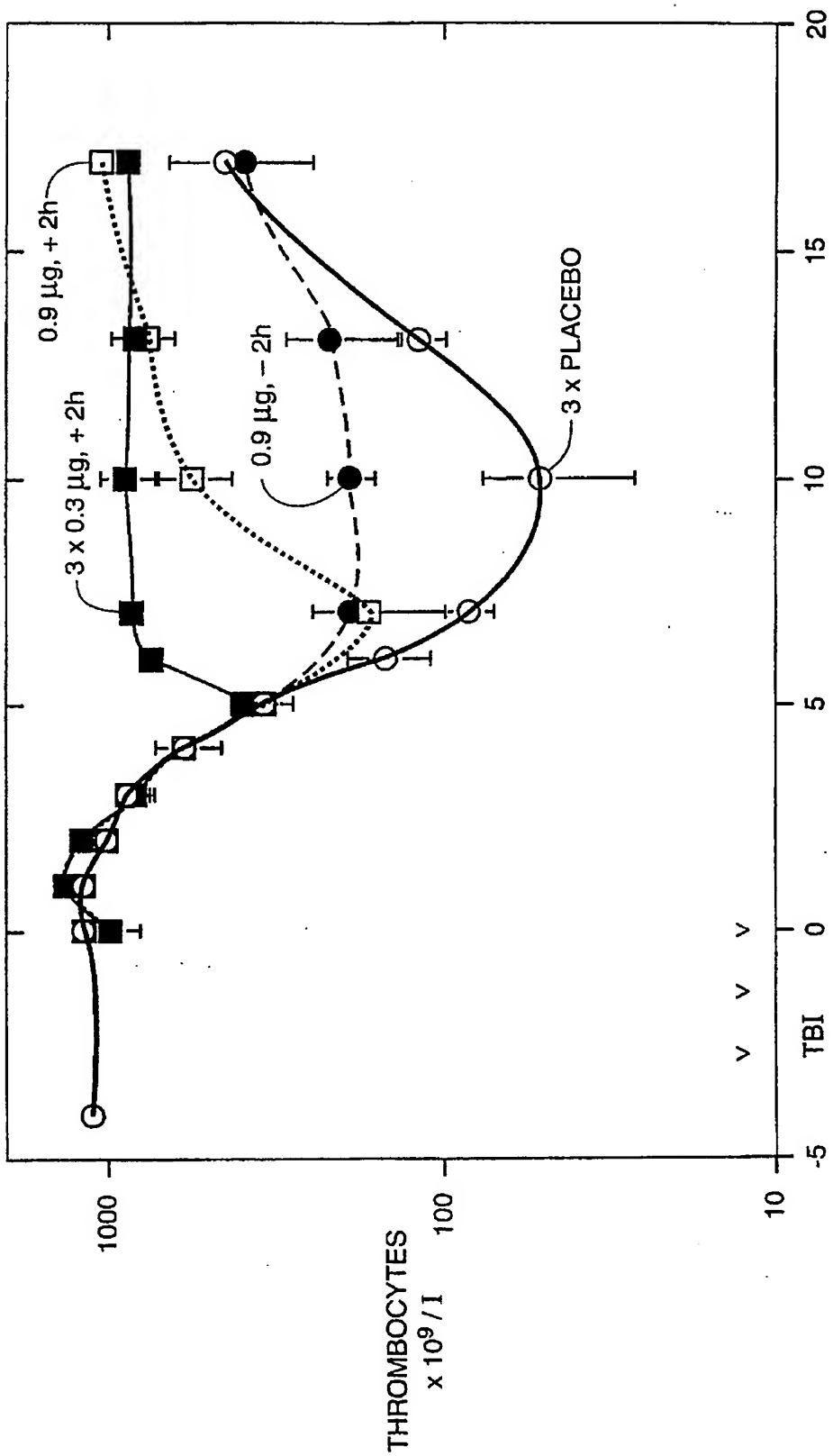


FIG._10

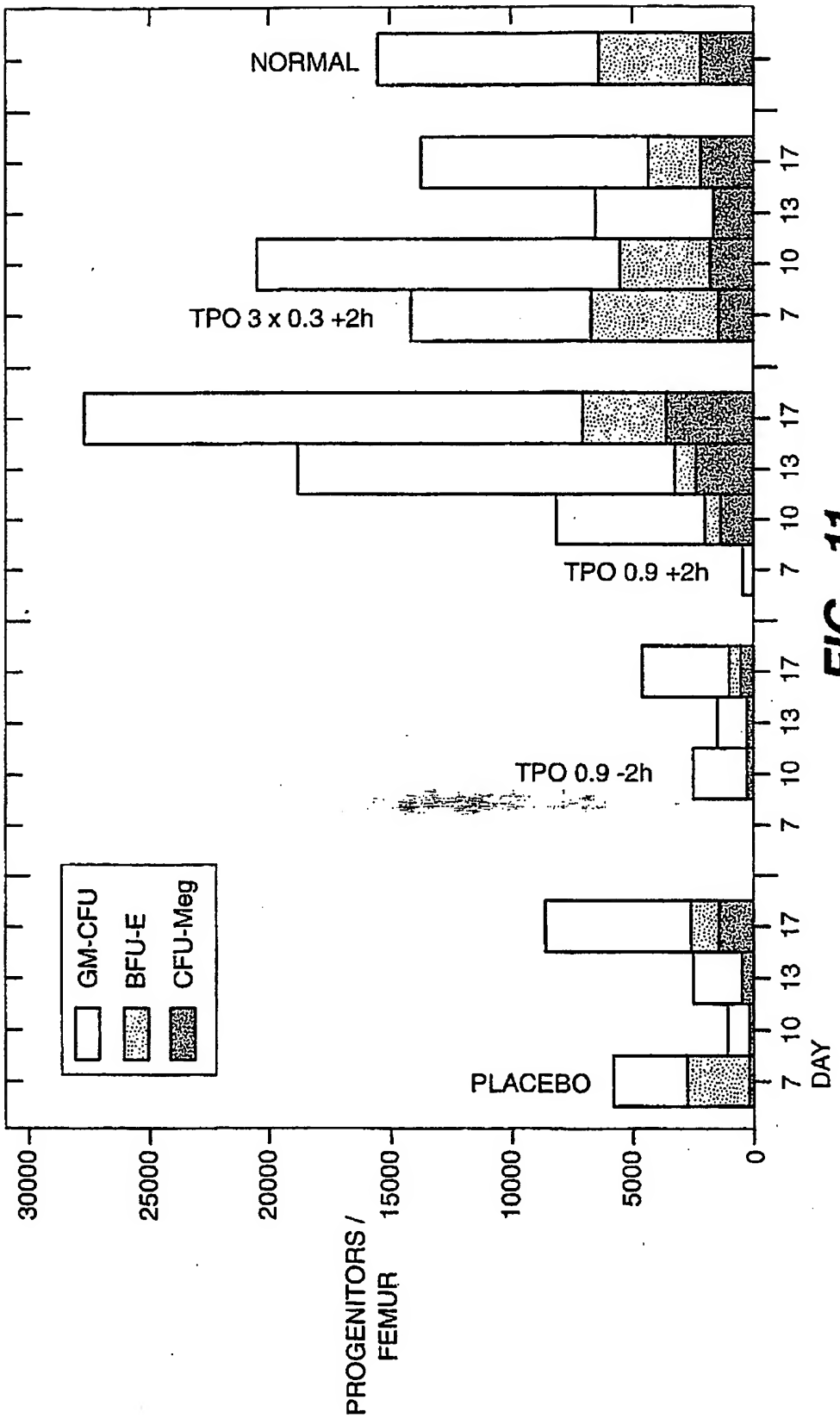

NORMAL
TPO 3 x 0.3 +2h
TPO 0.9 +2h
TPO 0.9 -2h
PLACEBO
GM-CFU
BFU-E
CFU-Meg
7 10 13 17
DAY
0
5000
10000
15000
20000
25000
30000
PROGENITORS / FEMUR

FIG._11

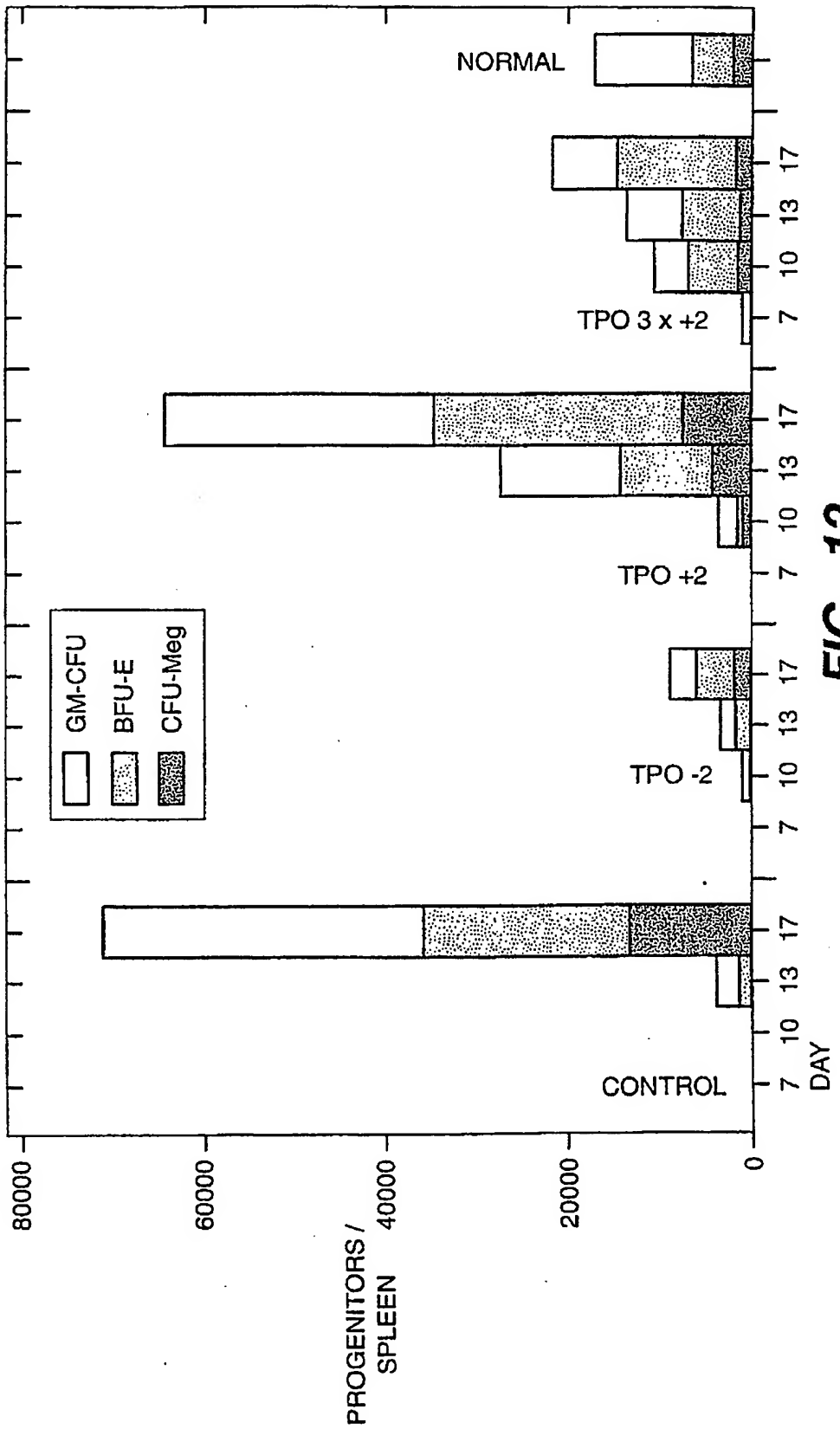
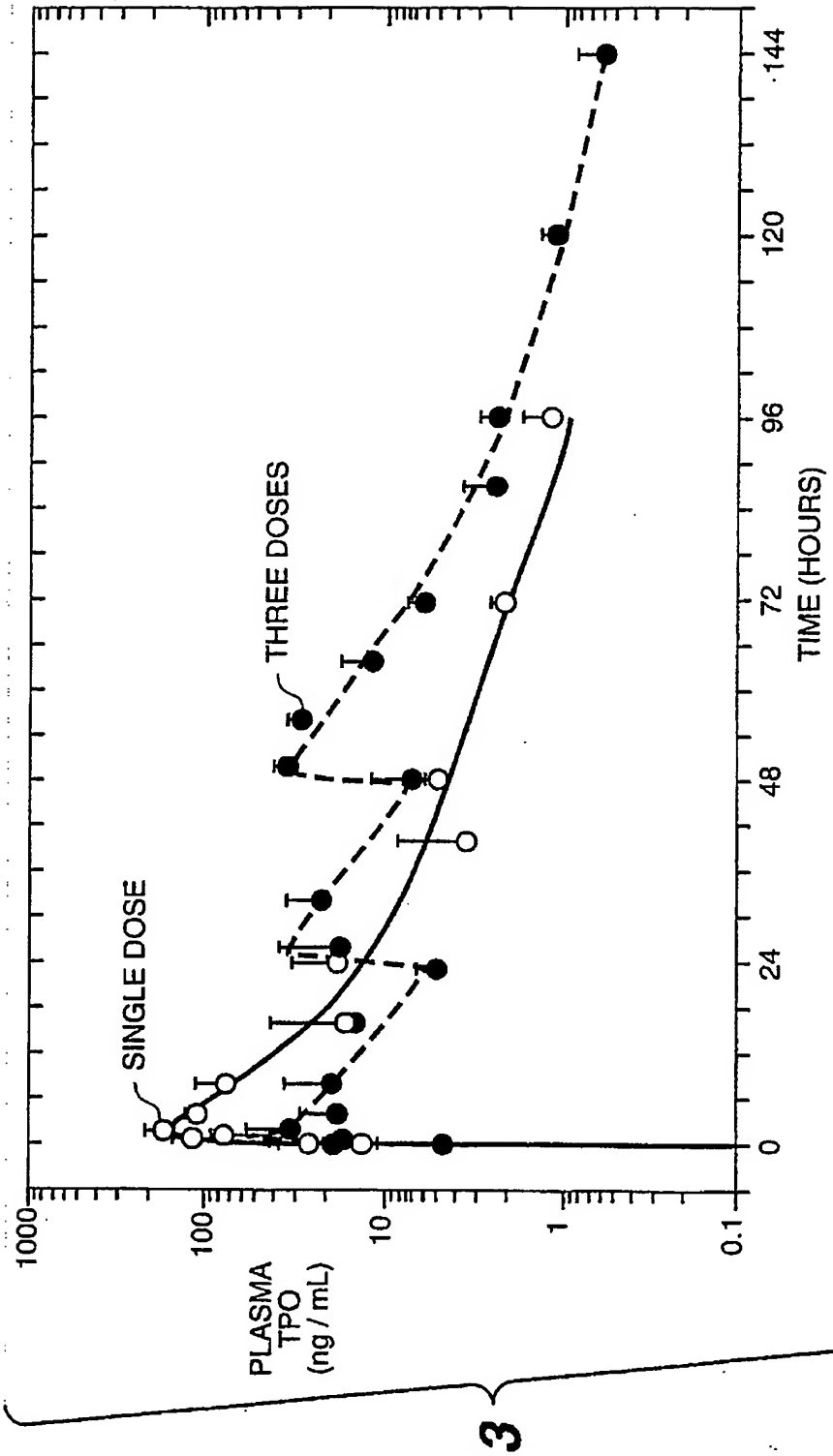

NORMAL
TPO 3 x +2
TPO +2
TPO -2
CONTROL
GM-CFU
BFU-E
CFU-Meg
7 10 13 17
DAY
0
20000
40000
60000
80000
PROGENITORS / SPLEEN

FIG._12



PK PARAMETERS	SINGLE DOSE	THREE DOSES
Cmax (ng / mL)	151	29.2
AUC $_{0\text{-tlast}}$ (ng / mL*hr)	1730	1420
$t_{1/2}$TERMINAL (hr)	21.1	35.0
CLEARANCE (mL / hr / kg)	26.5	27.2

FIG._13

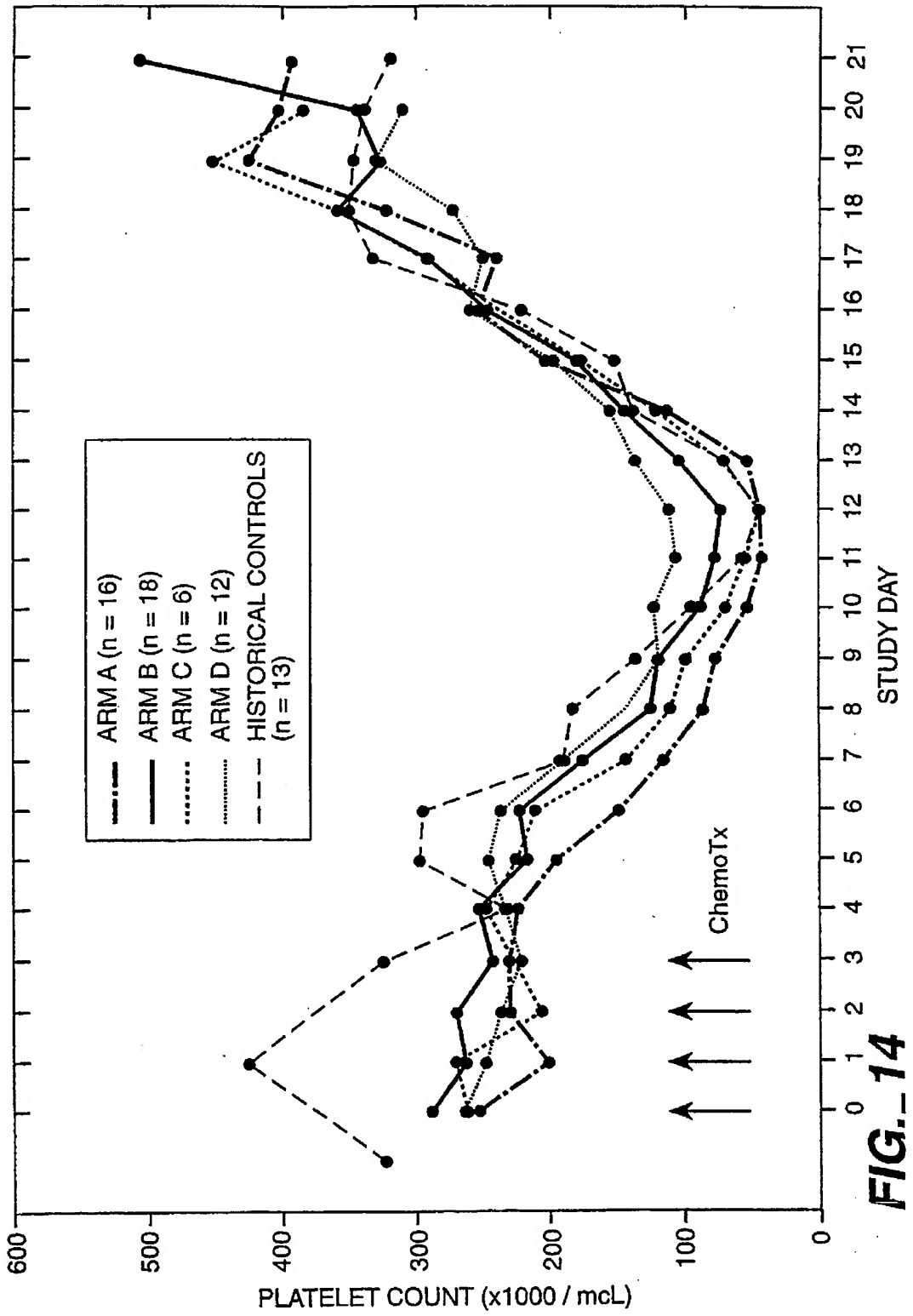

ARM A (n = 16)
ARM B (n = 18)
ARM C (n = 6)
ARM D (n = 12)
HISTORICAL CONTROLS (n = 13)
ChemoTx
PLATELET COUNT (x1000 / mcL)
STUDY DAY
0
100
200
300
400
500
600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

FIG._14

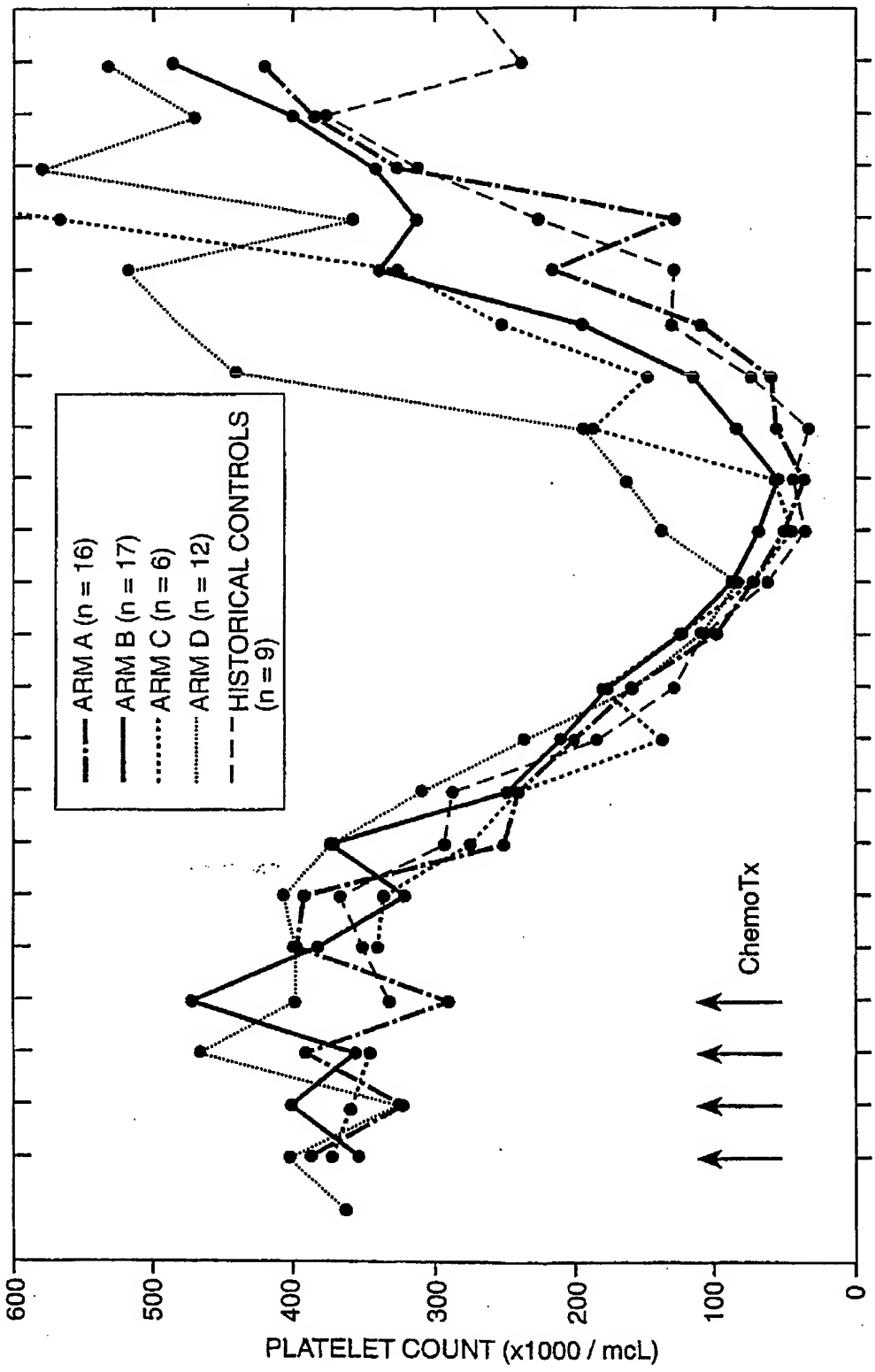

ARM A (n = 16)
ARM B (n = 17)
ARM C (n = 6)
ARM D (n = 12)
HISTORICAL CONTROLS (n = 9)
ChemoTx
PLATELET COUNT (x1000 / mcL)
STUDY DAY
0
100
200
300
400
500
600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

FIG._15

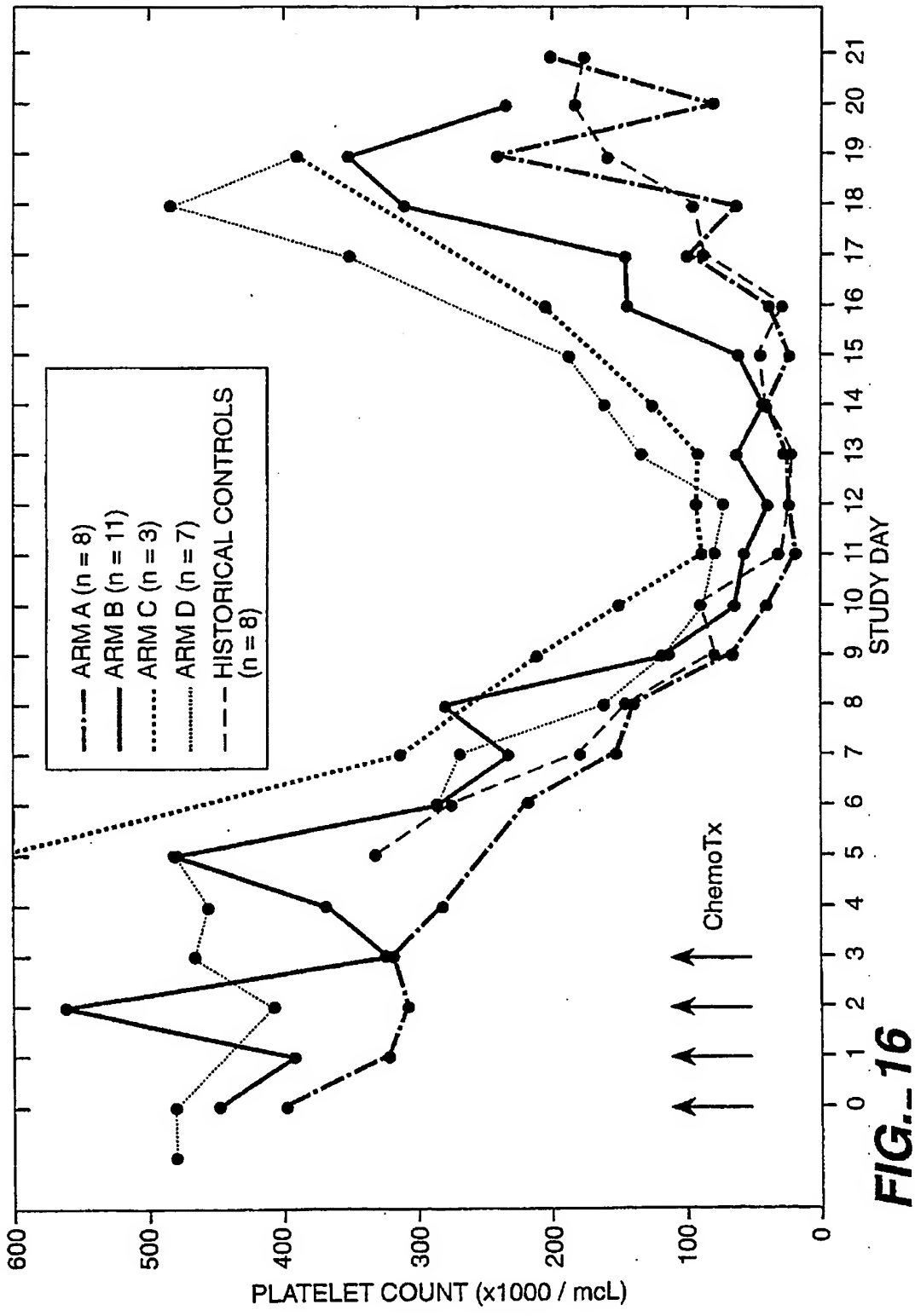

ARM A (n = 8)
ARM B (n = 11)
ARM C (n = 3)
ARM D (n = 7)
HISTORICAL CONTROLS (n = 8)
ChemoTx
PLATELET COUNT (x1000 / mcL)
STUDY DAY
0
100
200
300
400
500
600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

FIG._16

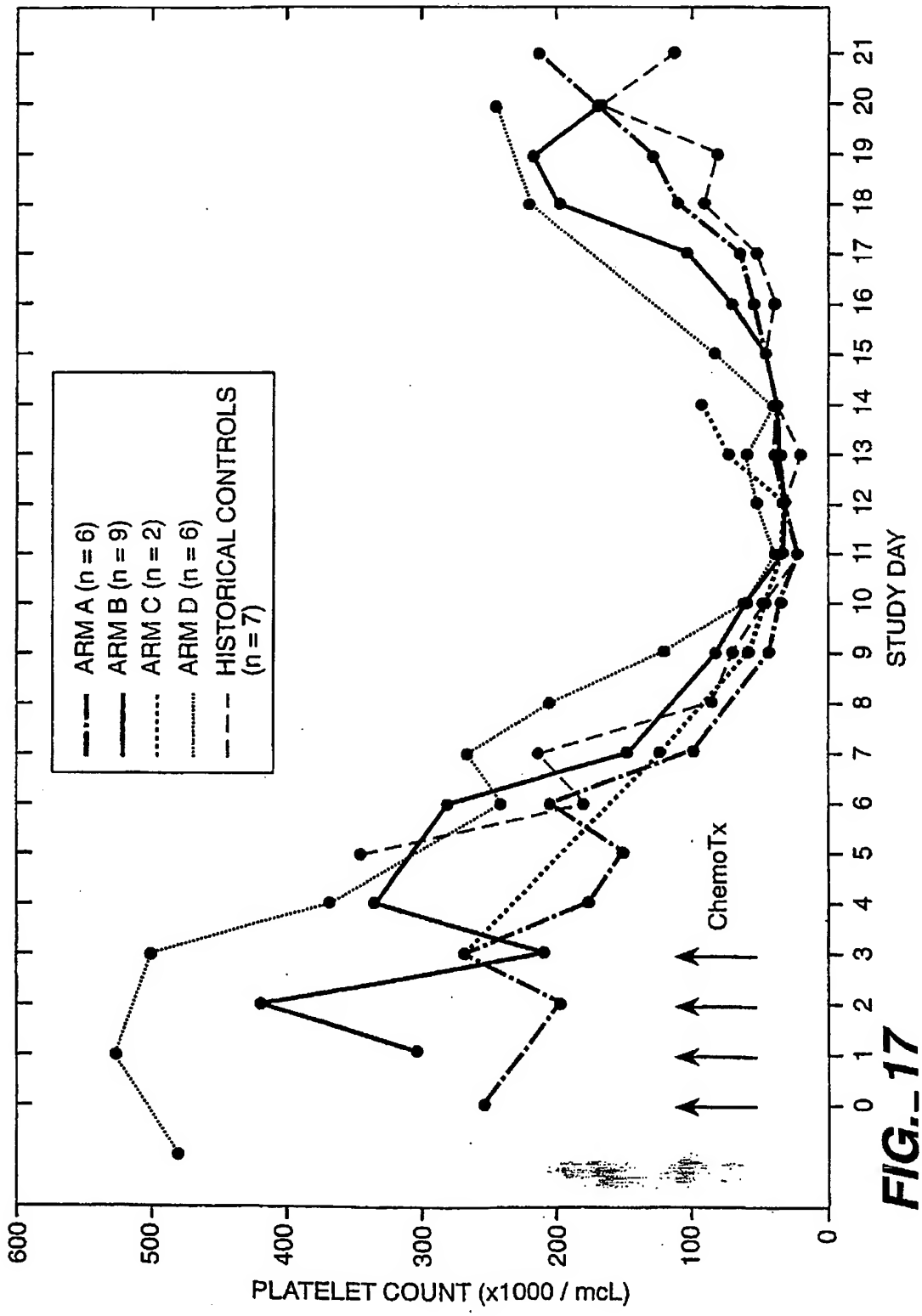


FIG._17

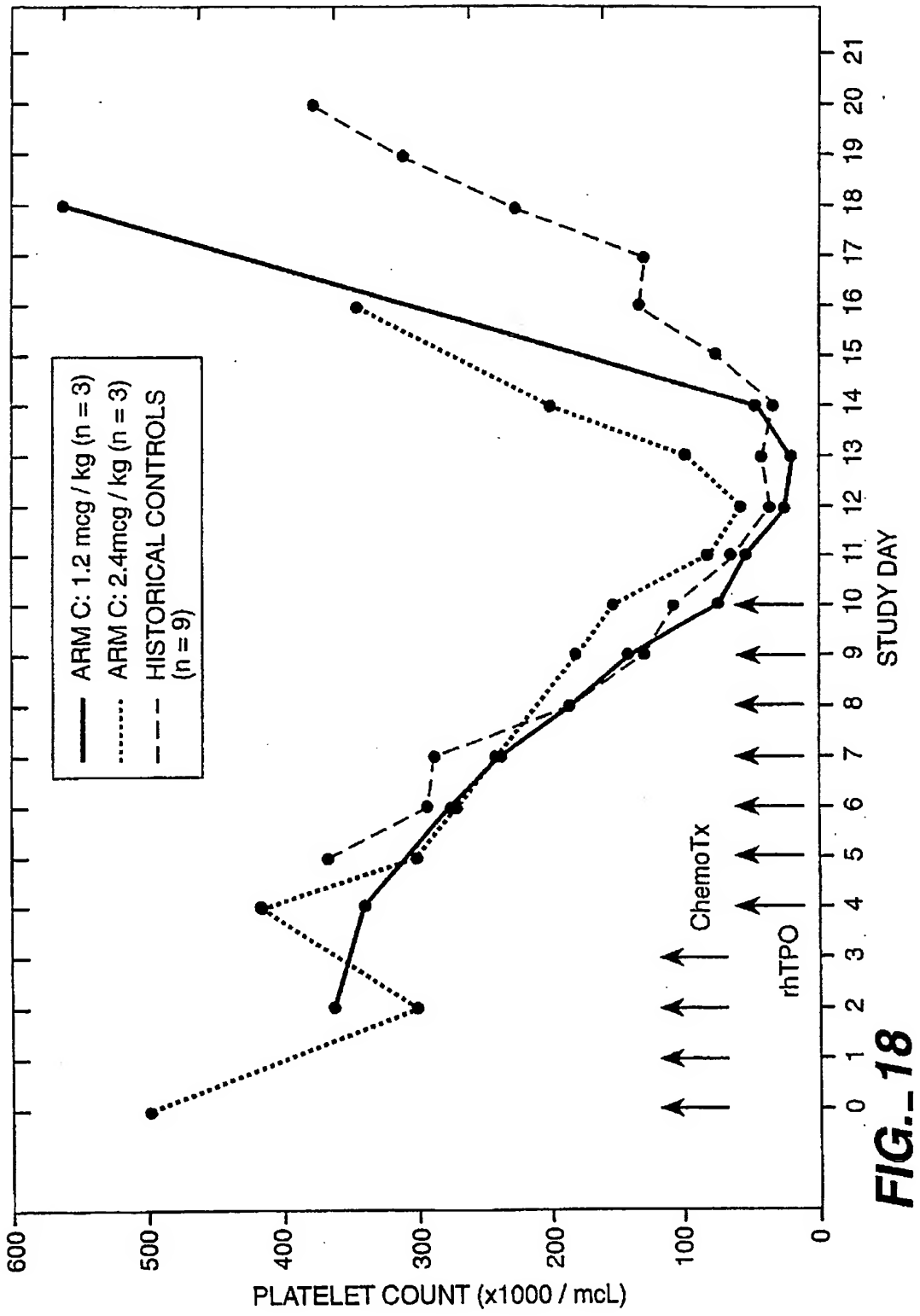

ARM C: 1.2 mcg / kg (n = 3)
ARM C: 2.4mcg / kg (n = 3)
HISTORICAL CONTROLS (n = 9)
PLATELET COUNT (x1000 / mcL)
600
500
400
300
200
100
0
STUDY DAY
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
rhTPO
ChemoTx

FIG._18

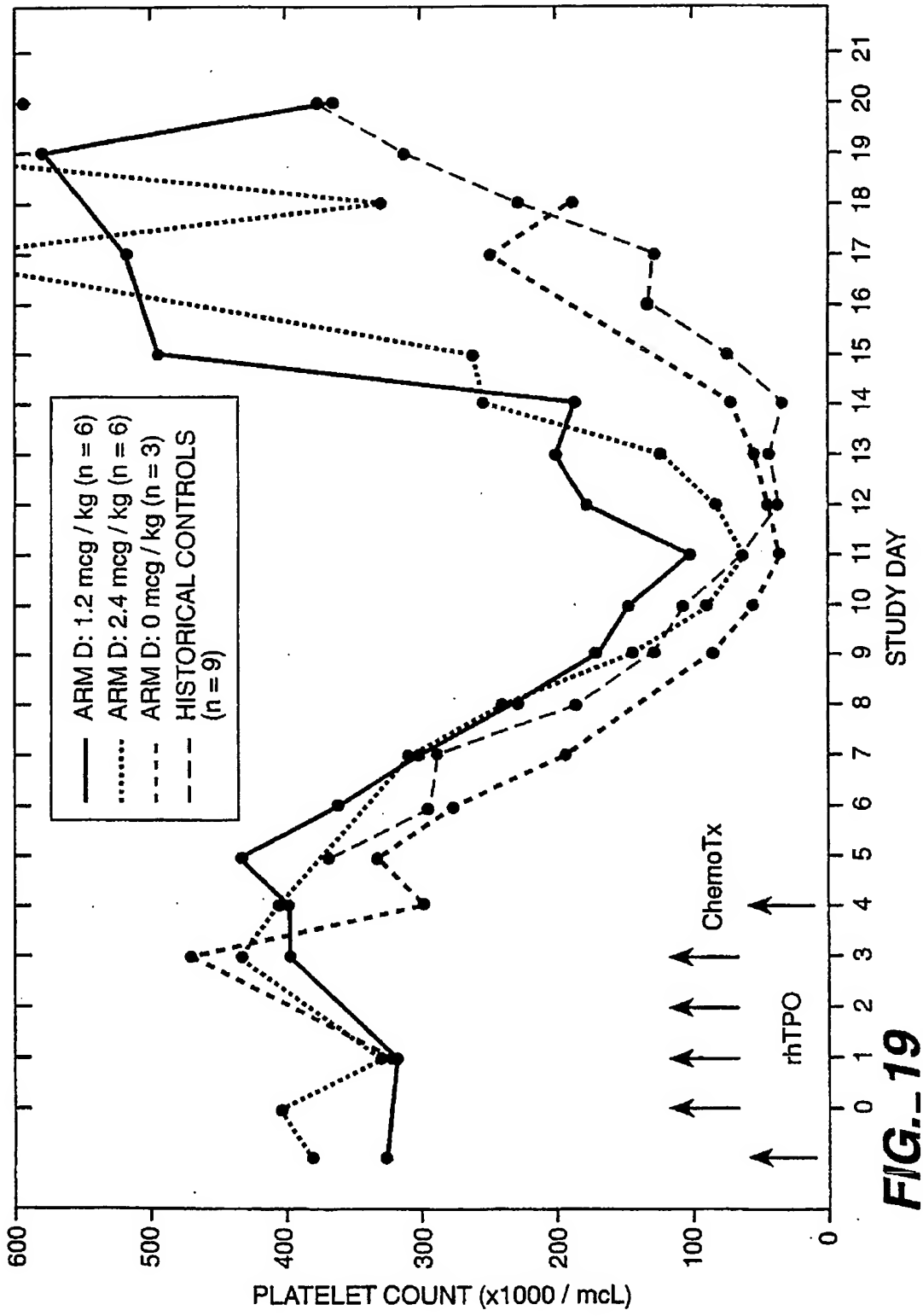

ARM D: 1.2 mcg / kg (n = 6)
ARM D: 2.4 mcg / kg (n = 6)
ARM D: 0 mcg / kg (n = 3)
HISTORICAL CONTROLS (n = 9)
PLATELET COUNT (x1000 / mcL)
600
500
400
300
200
100
0
STUDY DAY
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
rhTPO
ChemoTx

FIG._19

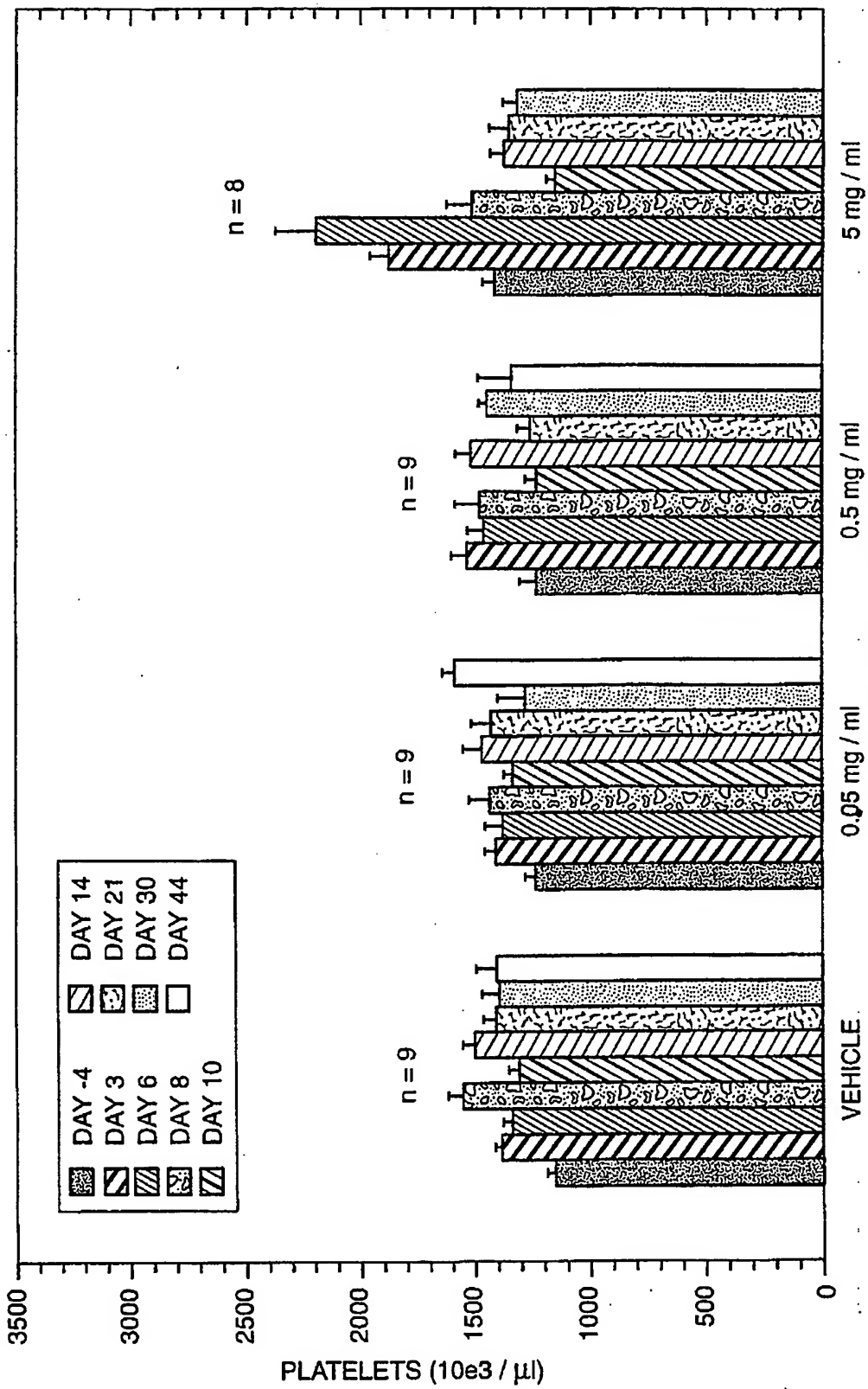

PLATELETS (10e3 / µl)
3500
3000
2500
2000
1500
1000
500
0
DAY -4
DAY 3
DAY 6
DAY 8
DAY 10
DAY 14
DAY 21
DAY 30
DAY 44
n = 9
n = 9
n = 9
n = 8
VEHICLE
0.05 mg / ml
0.5 mg / ml
5 mg / ml

FIG._20

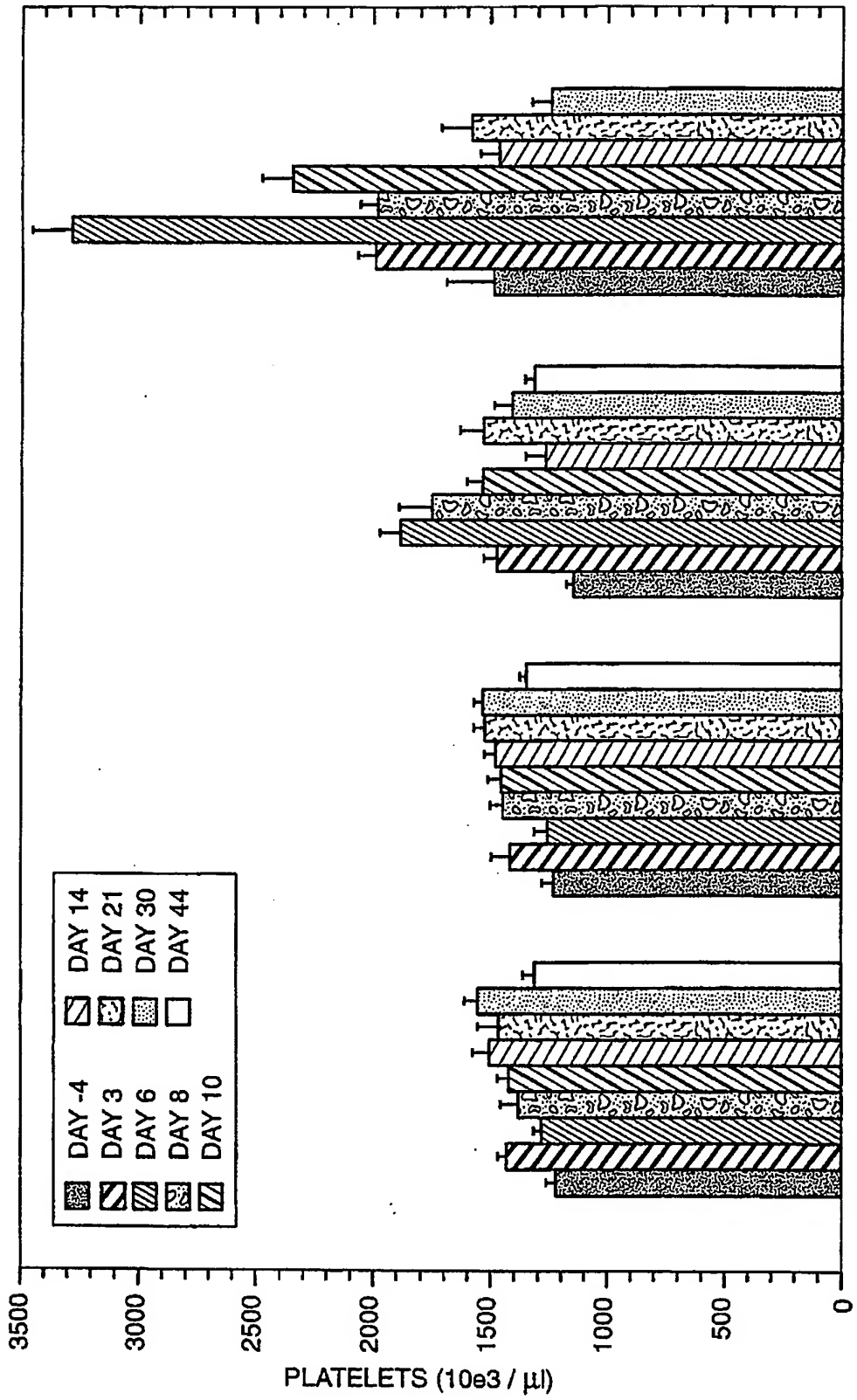

DAY -4
DAY 3
DAY 6
DAY 8
DAY 10
DAY 14
DAY 21
DAY 30
DAY 44
VEHICLE
0.05 mg / ml
0.5 mg / ml
5 mg / ml
PLATELETS (10e3 / µl)
3500
3000
2500
2000
1500
1000
500
0

FIG._21

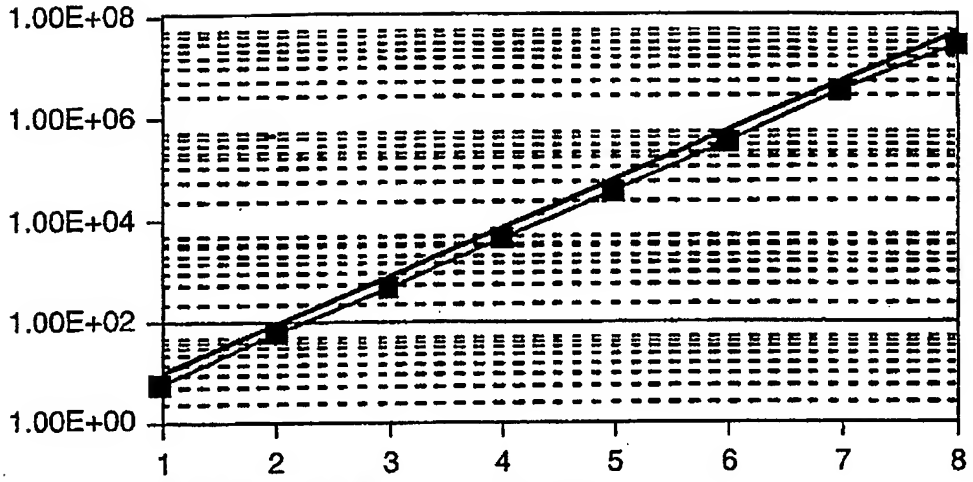


FIG._22

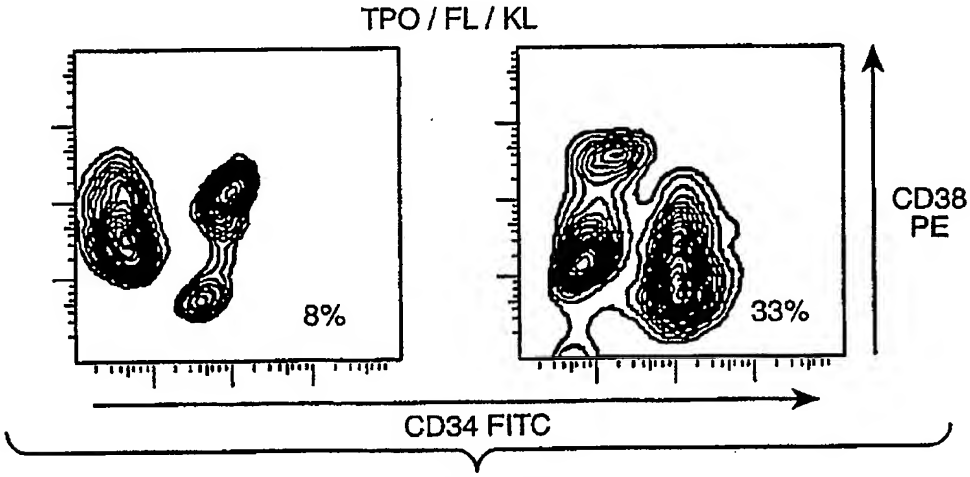


FIG._23

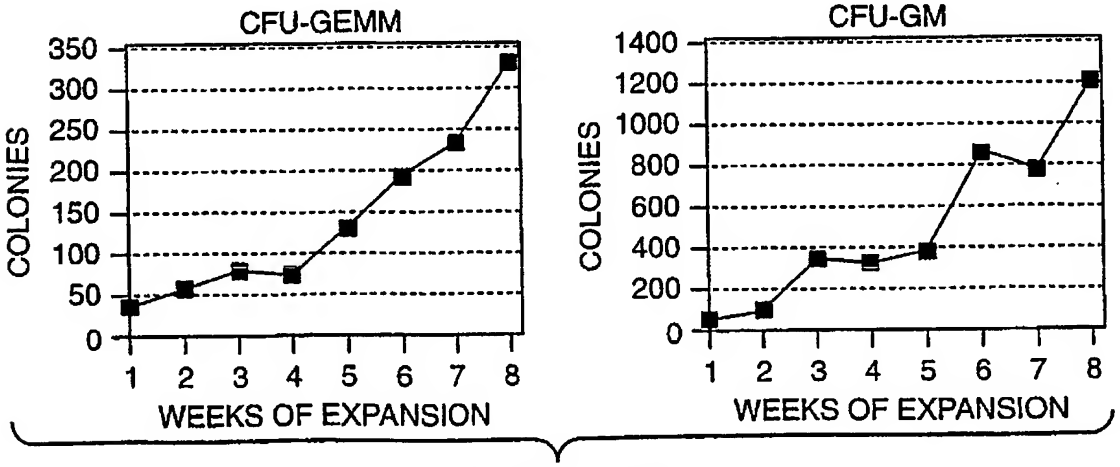


FIG._24

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 98/10475

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/19 A61K35/14

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BASSER R L ET AL: "Randomized, blinded, placebo -controlled phase I trial of pegylated recombinant human megakaryocyte growth and development factor with filgrastim after dose-intensive chemotherapy in patients with advanced cancer 'published erratum appears in Blood 1997 Sep 15;90(6):2513!." BLOOD, (1997 MAY 1) 89 (9) 3118-28, XP002076300 see the whole document	1-48
A	EP 0 675 201 A (AMGEN INC.) 4 October 1995 see the whole document	1-48
P,X	WO 97 26907 A (GENENTECH INC.) 31 July 1997 see the whole document	1-48

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
2 September 1998	21/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 98/10475

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	NEELIS K J ET AL: "Prevention of thrombocytopenia by thrombopoietin in myelosuppressed rhesus monkeys accompanied by prominent erythropoietic stimulation and iron depletion." BLOOD, (1997 JUL 1) 90 (1) 58-63, XP002076301 see the whole document	1-48
P,X	NEELIS K J ET AL: "The efficacy of single-dose administration of thrombopoietin with coadministration of either granulocyte/macrophage or granulocyte colony-stimulating factor in myelosuppressed rhesus monkeys." BLOOD, (1997 OCT 1) 90 (7) 2565-73, XP002076302 see the whole document	1-48
P,X	NEELIS K J ET AL: "Simultaneous administration of TPO and G-CSF after cytoreductive treatment of rhesus monkeys prevents thrombocytopenia, accelerates platelet and red cell reconstitution, alleviates neutropenia, and promotes the recovery of immature bone marrow cells." EXPERIMENTAL HEMATOLOGY, (1997 SEP) 25 (10) 1084-93, XP002076303 see the whole document	1-48

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In .iational application No.
PCT/US 98/ 10475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-44, 47-48 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/10475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 675201 A	04-10-1995	US 5795569 A	18-08-1998
		AT 169335 T	15-08-1998
		AU 691606 B	21-05-1998
		AU 2230895 A	23-10-1995
		BG 100625 A	30-04-1997
		BR 9506017 A	14-10-1997
		CN 1137757 A	11-12-1996
		CZ 9503505 A	15-01-1997
		EP 0690127 A	03-01-1996
		EP 0755263 A	29-01-1997
		FI 960136 A	11-03-1996
		HU 74257 A	28-11-1996
		LV 11783 A	20-06-1997
		LV 11783 B	20-12-1997
		NO 960111 A	24-09-1996
		PL 312577 A	29-04-1996
		SK 166695 A	05-02-1997
		WO 9526746 A	12-10-1995
		US 5766581 A	16-06-1998
		ZA 9502651 A	25-06-1996
		ZA 9502652 A	21-12-1995
WO 9726907 A	31-07-1997	AU 1533597 A	20-08-1997

[Patent Search]

Title: Process for separating megakaryocyte stimulating factor from human plasma and purifying it and its application			
Application Number:	02112722	Application Date:	2002.03.06
Publication Number:	1369507	Publication Date:	2002.09.18
Approval Pub. Date:		Granted Pub. Date:	2004.10.06
International Classification:	A61K38/18,A61P7/06,C07K14/475,C12N5/08		
Applicant(s) Name:	Nanjing Univ		
Address:	210093		
Inventor(s) Name:	Zhang Heyun, Jin Yifeng		
Attorney & Agent:	hu tiyu		

Abstract

A process for separating megakarylcyte stimulator (MES) from human plasma and purifying it includes dissolving the deposite of human plasma, which is generated by extracting albumin and globulin centrifugal treating, ultrafiltrating, ion-exchange cellulose chromatography, gel filter and DEAE gel chromatography. The said MES has 40-42 Kda or 18-20 kda of molecular weight, and can promote the generation of thrombocytes and the multiplication of naval blood's stem cells, so it can be used for treating thrombocytopenia and stem cell transplant.

[19]中华人民共和国国家知识产权局

[51]Int. Cl[7]
C07K 14/475
A61K 38/18 A61P 7/06
C12N 5/08

[12]发明专利申请公开说明书

[21]申请号 02112722.0

[43]公开日 2002年9月18日

[11]公开号 CN 1369507A

[22]申请日 2002.3.6 [21]申请号 02112722.0
[71]申请人 南京大学
地址 210093 江苏省南京市汉口路22号
[72]发明人 张鹤云 金以丰 张太平
汤国枝 袁达文
[74]专利代理机构 南京知识律师事务所
代理人 胡锡瑜
权利要求书1页 说明书6页 附图页数0页

[54]发明名称 从人血浆中分离纯化巨核细胞刺激因子的方法及应用

[57]摘要

本发明属于生化分离纯化技术领域。本发明从人血浆经生产白蛋白和球蛋白后的沉淀物中，通过溶解、离心、超滤、离子交换纤维素层析，凝胶过滤，DEAE凝胶层析等方法，分离纯化了巨核细胞刺激因子(Megakarylcyte Stimulator，简称MES)，MES具有分子量40－42Kda和18－20Kda两种形式，等电点4.6±0.1，均具有促进巨核细胞的祖细胞分化成熟，表现出巨核细胞的功能，从而可以在体内促进血小板生成，对血小板低下症具有专一的治疗效果。此外MES还具有促进脐带血干细胞增殖的功能。在干细胞移植中有着重大的潜在价值。

ISSN1008-4274

BAD ORIGINAL

[illegible]

一、发明领域：

[illegible]

二、背景技术

[illegible]

[illegible]

[illegible]

[illegible]

三、发明内容：

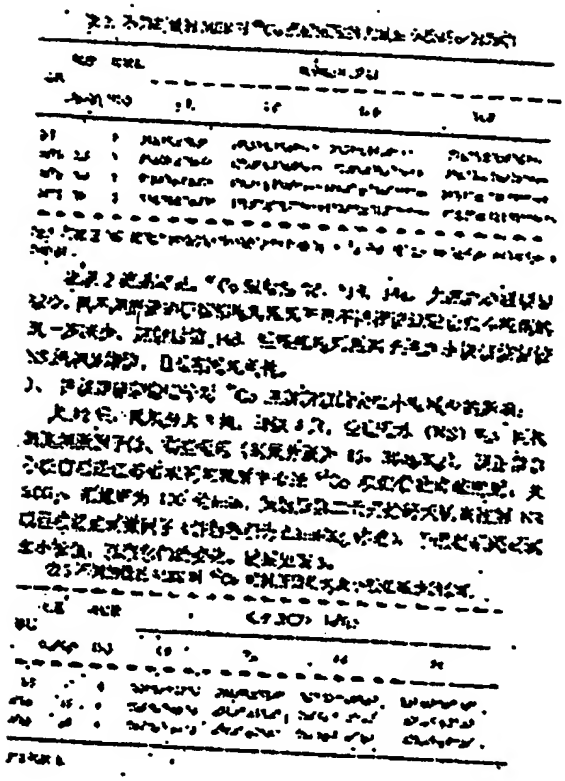
[illegible]

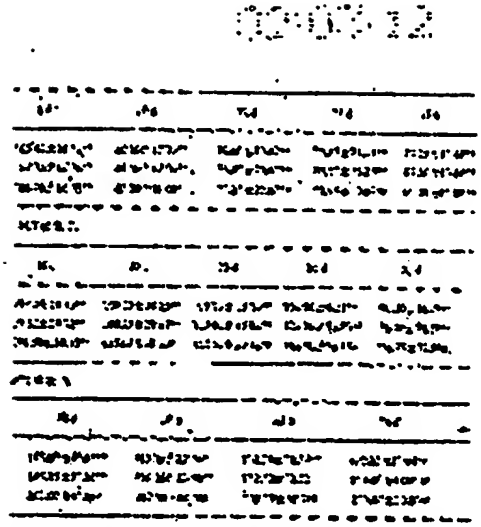
[illegible]

[illegible]

[illegible]

BAD ORIGINAL





BAD ORIGINAL